Phytosterols Affect Endocrinology and Metabolism of the Field Vole (*Microtus agrestis*)

PETTERI NIEMINEN,[1]*‡ ANNE-MARI MUSTONEN,* PIRJO LINDSTRÖM-SEPPÄ,†
VIRVE KÄRKKÄINEN,*† HELENA MUSSALO-RAUHAMAA,‡ AND JUSSI V.K. KUKKONEN*

Department of Biology, University of Joensuu, FIN-80101 Joensuu, Finland; †Department of Physiology, University of Kuopio, FIN-70211 Kupio, Finland; and ‡Department of Public Health, University of Helsinki, FIN-00014 Helsinki, Finland

Phytosterols or plant sterols (PS) enter the ecosystem via pulp mill effluents. They are also consumed by the general population of developed countries in natural remedies and margarines to lower elevated serum cholesterol levels. This study screened the endocrine and enzymatic parameters of the field vole (*Microtus agrestis*) for the effects of subchronic PS exposure at three doses (0, 5, or 50 mg of PS kg^{-1} day^{-1}). PS at 5 or 50 mg kg^{-1} day^{-1} decreased the relative liver weight of the voles. The kidney glycogen phosphorylase activity decreased at 5 or 50 mg kg^{-1} day^{-1}, but the liver glycogen phosphorylase activity increased at 5 mg kg^{-1} day^{-1}. The plasma estradiol and testosterone concentrations of males were higher due to PS supplement at 5 mg kg^{-1} day^{-1}. This can be due to increased sex steroid synthesis from PS precursors. Biotransformation enzyme activities were not affected. PS caused multiple, previously unreported effects that were more pronounced at a low dose. As 5 mg PS kg^{-1} day^{-1} is the recommended dose for various health products, a thorough risk assessment of the effects and interactions of PS is warranted. Exp Biol Med 228:188–193, 2003

Key words: endocrine disruption; estradiol; field role; *Microtus agrestis*; phytosterols; testosterone

Phytosterols or plant sterols (PS) are analogs of animal cholesterol in plants. Due to their cholesterol-lowering effects, PS are used for the treatment of hypercholesterolemia and cardiovascular diseases and their risk factors (1, 2). About 5% of ingested PS are absorbed in humans (3). The absorption of β-sitosterol is about 5%, and that of sitostanol close to 0%. In rats, the absorption of β-sitosterol is about 4%, and the absorption of sitostanol is 1% (4). Plasma PS levels are usually between 7 and 24 μM l^{-1}, which is less than 5% of the total sterol concentration in human plasma. In the western diet, the daily intake of PS is about 80 mg. However, in the vegetarian or Japanese diet, the intake can be as high as 400 mg day^{-1} (3).

PS and stanol esters lower circulating total and low-density lipoprotein (LDL) cholesterol levels by suppressing cholesterol absorption in the intestine at a dose of 1.84 g day^{-1} (5) (approximately 26 mg of PS kg^{-1} day^{-1} for a 70-kg person). The addition of β-sitosterol to a diet results in a 42% decrease in cholesterol absorption (6). In fact, PS are added to margarines at a dose of 30 mg kg^{-1} day^{-1} to lower elevated cholesterol levels (7). There is also widespread use of PS in various commercial health products, where the recommended dose is 300 mg day^{-1} (for review, see Ref. 3) or 4–5 mg kg^{-1} day^{-1} for a 70-kg person.

Dietary PS also have possible anticancer effects. β-Sitosterol may offer protection against breast cancer by stimulating apoptosis and by inhibiting tumor growth in cancer tissue (8). The growth of human prostate cancer LNCaP cells can be inhibited by PS (9) and PS may also protect from dietary-induced colon cancer in rats (10).

Pulp mill effluents are a source of PS in nature causing reduced sex steroid levels and gonad size in fish (11, 12). β-Sitosterol reduces reproductive fitness in the goldfish (*Carassius auratus*) by decreasing testosterone and 11-ketotestosterone levels in males and testosterone and 17-β-estradiol levels in females (13). β-Sitosterol also induces vitellogenin gene expression in the rainbow trout (*Oncorhynchus mykiss*) (12). In rats, subcutaneous β-sitosterol at 0.5–5 mg kg^{-1} day^{-1} reduces sperm count and testicular weight (3).

The field vole (*Microtus agrestis* L., 1761) is a small rodent common in Scandinavia and Central Europe. The body mass of an adult female field vole is about 35 g and an adult male is 40 g (14). Both males and females reach sexual maturity at the age of 50–60 days. The reproductive period of the species is between April and September. The female gives birth to a litter of up to five individuals after a 20-day pregnancy (15).

This study is part of a research project studying the effects of plant-derived and chemical endocrine disruptors on carnivores and herbivores of natural stock. The field vole was chosen as a representative of a common small herbi-

This work was supported by TEKES (National Technology Agency of Finland).
[1] To whom requests for reprints should be addressed at Department of Biology, University of Joensuu, P.O. Box 111, FIN-80101 Joensuu, Finland. E-mail: pniemine@cc.joensuu.fi

Received April 1, 2002.
Accepted October 11, 2002.

1535-3702/03/2282-0188$15.00

vore. As the field vole is very common, it has potential as a possible bioindicator species to study the effects of endocrine disruptors in natural ecosystems. The aim of the present study was to investigate the subacute effects of PS on endocrine parameters and phase I and II biotransformation enzymes. This study also aimed to select from the possible diverse effects of PS the most susceptible parameters to be used as biomarkers and start points for further study.

Materials and Methods

Animals and Exposure. For this study, 31 field voles (14 males and 17 females) were randomly assigned into three study groups. The animals were from the breeding colony of the University of Joensuu. Their parents had been captured in the wild (Punkaharju, Finland, 61°N), and the experimental animals were of the first generation born in captivity. The animals were born between October 1, 2000 and March 18, 2001. The voles of different age were assigned to their respective study groups in a random fashion. The animals were housed singly in solid bottom plastic cages (Makrolon: 42 × 22 × 15 cm) with wood shavings for bedding and free access to water and a pelleted diet (Avelsfoder för råtta och mus R36: protein 18.5% and fat 4.0%, energy content 1260 kJ 100 g^{-1}; Lactamin, Stockholm, Sweden). The Animal Care and Use Committee of the University of Joensuu approved all procedures.

The study period was from May 9 to May 23, 2001. The first group of three males and four females was the control group. The other groups were given Ultra-sitosterol (88.7% β-sitosterol + β-sitostanol, 0.0% α-sitosterol, 9.0% campesterol + campestanol, 0.9% artenols; UPM Kymmene, Kaukas, Lappeenranta, Finland) daily perorally mixed with the same feed that the control group received. The second group of four males and seven females received PS at a dose of 5 mg kg^{-1} day^{-1} and the third group of seven males and six females received PS at a dose of 50 mg kg^{-1} day^{-1}. The feed was palatable to the animals and no macroscopic adverse effects could be seen in the overall well being of the animals (activity, feeding, and locomotion) or in the organs at necropsy.

After 2 weeks, the animals were sacrificed quickly with diethyl ether. The amount of feed eaten during the whole study period was measured. The animals were weighed, their length was measured, and their sex was reconfirmed intraabdominally. Blood samples were obtained with cardiac punctures with sterile needles and syringes into test tubes containing EDTA and were centrifuged at 4000*g* to obtain plasma. The livers, testes, and kidneys were dissected and weighed. All the samples were frozen in liquid nitrogen and stored at −80°C.

Hormone Assays. Hormone concentrations were measured using the radioimmunoassay (RIA) and immunoradiometry methods. The plasma tetraiodothyronine (T_4) and testosterone concentrations were measured using the Spectria [^{125}I]-Coated Tube Radioimmunoassay kits (Orion Diagnostica, Espoo, Finland). The plasma luteinizing hormone (LH) and thyroid-stimulating hormone (TSH) concentrations were measured using the Spectria-Coated Tube Immunoradiometric Assay kits (Orion Diagnostica). The plasma leptin levels were measured with the Multi-Species Leptin RIA kit (Linco Research, St. Charles, MO). The plasma ghrelin levels were determined with the Ghrelin (human) RIA kit (Phoenix Pharmaceuticals, Belmont, CA). The plasma estradiol concentrations were determined using the enzyme-linked immunoassay (ELISA) method (17β-Estradiol Immunoassay; R&D Systems, Wiesbaden-Nordenstadt, Germany). Cross-reactivity to estrone was 4.64% and was 0.53% to estriol; cross-reactivity to all other steroids was <0.06% (manufacturer's specification report). The sex steroid assays were validated such that serial dilutions of the field vole plasma showed linear changes in OD values that were parallel with the standard curve produced with human standards. The PS mixture used in the experiments at a concentration as great as 100 mg ml^{-1} did not produce a positive OD reading in the ELISA assay for estradiol (data not shown). The LH, TSH, leptin and ghrelin measurements were carried out by pooling 5–10 μl of plasma from each animal of a dose group together, as there would have been an insufficient amount of plasma for the measurements from single animals (100 μl was required for e.g., the leptin measurement, about 50 μl was obtained per animal). The determinations of plasma leptin, ghrelin, LH, and TSH in the field vole have been reported previously (16). For the quantitation of radioactivity in the RIA, a 1470 Wizard Gamma Counter (Wallac, Turku, Finland) was used.

Measurement of Enzyme Activities. The liver and kidney samples were weighed and homogenized in cold citrate buffer at pH 6.5 for the glucose-6-phosphatase (G6Pase) determination, at pH 6.1 for the glycogen phosphorylase measurement, and in cold 0.85% sodium chloride for the lipase esterase measurement. The activity of G6Pase was measured using G6Pase as substrate in the presence of EDTA after an incubation time of 30 min at 37.5°C (17). The glycogen phosphorylase activity was measured in the presence of glucose-1-phosphate, glycogen, sodium fluoride, and AMP according to the method of Hers and van Hoof (17). The lipase esterase activity was measured according to the method of Seligman and Nachlas (18) using as substrate 2-naphtyl-laurate without taurocholate. Glycogen concentrations in the liver and kidney were measured spectrophotometrically according to the method of Lo *et al.* (19).

For the preparation of microsomes, the liver samples were thawed out and homogenized in ice-cold 0.25 *M* sucrose, pH 7.4, using 4 ml of solution g^{-1} liver wet weight. After homogenization (eight strokes at 1160 rpm), the livers were centrifuged at 10,000*g* for 20 min. The supernatants were centrifuged at 105,000*g* for 60 min to pellet the microsomes. The pellets were then resuspended in 0.25 *M* sucrose, pH 7.4, to a final volume of 1 ml g^{-1} tissue. The microsomes were stored at −80°C.

For the enzyme analysis, the hepatic monooxygenase activity was determined according to Burke and Mayer (20) from the microsomal fraction using 7-ethoxyresorufin as substrate. The *O*-deethylation of 7-ethoxyresorufin (EROD) was measured with a fluorescence spectrophotometer (RF-5001PC; Shimadzu, Columbia, MD) in a kinetic reaction with resorufin as an internal standard. In the reaction mixture in the cuvette, 1.97 ml of 0.05 *M* TRIS-HCl buffer (pH 7.5) containing 0.025 *M* $MgCl_2$ and 7-ethoxyresorufin (final concentration of 1 μM) was mixed with 20 µl of microsomes. The reaction was started with 10 µl of 10 m*M* NADPH, and was then followed for 2 min. The microsomal UDP-glucuronosyltransferase (UDPGT) activity was measured spectrophotometrically (UV-240; Shimadzu) as described by Hänninen (21). The incubation mixture (total volume of 150 µl) contained 0.35 m*M* *p*-nitrophenol as aglycone and 4.5 m*M* UDP-glucuronic acid in the presence of 20 m*M* K_2EDTA. The enzyme concentration was about 1 mg of microsomal protein. The reaction was stopped with 3% trichloroacetic acid. The aliquot was alkalinized using 5 *N* NaOH, and the absorbance was measured at 400 nm.

The cytosolic glutathione *S*-transferase (GST) activity was analyzed by the kinetic method of Habig *et al.* (22) with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. The reaction mixture was contained as final concentrations of 0.1 *M* potassium phosphate buffer, pH 6.5, 1 m*M* glutathione, 1 m*M* CDNB, and about 0.1 mg of cytosolic protein. The reaction was followed in a spectrophotometer (Lambda 2 UV/VIS; Perkin-Elmer, Norwalk, CT) at 340 nm. The enzyme analyses were carried out at 37°C. All enzyme reactions were controlled to be linear with time and enzyme concentration. The protein content in microsomal and supernatant fractions was measured according to the method of Bradford (23).

Statistical Analyses. Multiple comparisons were performed with the two-way analysis of variance (ANOVA) when there were differences between the sexes and with the one-way ANOVA if the results of the males and females did not differ from each other. The *post hoc* Duncan's test was used to further evaluate significant effects. Paired comparisons were performed with the Student's *t* test. For nonparametric data, the Mann-Whitney *U* test was used. The $P < 0.05$ level was considered to be statistically significant. The normality of distribution and the homogeneity of variances were determined with the Kolmogorov-Smirnov test and with the Levene test. The correlations were calculated using the Spearman's correlation coefficient (r_s). The results are expressed as mean ± SE. If there was no sexual dimorphism within a variable, the results of the females and the males are pooled together in the "Results" section and in the tables.

Results

PS increased the food intake of the voles at 50 mg kg^{-1} day^{-1} (Mann-Whitney *U* test, $P < 0.003$, Table I). However, this did not affect the body mass or weight change of the animals. As expected, the males were heavier than the females (*t* test, $P < 0.03$). Absolute liver weights were also higher in the males than in the females (*t* test, $P < 0.05$), but the relative kidney weights were higher in the females (*t* test, $P < 0.001$). PS caused a decrease in the relative liver weights when both sexes were analyzed together at 5 or 50 mg kg^{-1} day^{-1} (*t* test, $P < 0.05$). The absolute testicular weight was slightly higher at 5 mg kg^{-1} day^{-1} compared with the control males (*t* test, $P < 0.06$).

PS caused biphasic effects on the liver carbohydrate and lipid metabolism. The liver G6Pase and glycogen phosphorylase activities were the highest at 5 mg kg^{-1} day^{-1} (ANOVA, $P < 0.05$, Table II). However, the kidney glycogen phosphorylase activity decreased due to PS treatment (ANOVA, $P < 0.05$). The lipase esterase activities in the liver and kidney were not affected, nor were there any effects of PS on the kidney G6Pase activities or on the liver and kidney glycogen contents.

The plasma estradiol concentrations of male voles were the highest at 5 mg kg^{-1} day^{-1} compared with the control group or the 50 mg kg^{-1} day^{-1} group (two-way ANOVA, $P < 0.05$, Table III). In the females, this increase at 5 mg kg^{-1} day^{-1} was nonsignificant. The effect could be observed in the plasma testosterone concentrations of male voles also at 5 mg PS kg^{-1} day^{-1} (two-way ANOVA, $P < 0.05$, Table III). However, the plasma LH concentration measured from pooled plasma was the lowest at 5 mg kg^{-1} day^{-1}. PS did not cause any changes in the plasma T_4 concentrations. However, the TSH values of the PS exposed groups measured from pooled plasma were almost below the detection limit and considerably lower (more than 60-fold) than the TSH value of the control animals.

The plasma ghrelin levels also showed a biphasic response with the lowest value at 5 mg kg^{-1} day^{-1} (Table III). In the plasma leptin levels, the result was the opposite, with the highest value at 5 mg kg^{-1} day^{-1}. As these hormone concentrations were measured from pooled plasma, these results need reconfirmation in future studies.

There were no differences in the liver EROD, UDPGT, or GST activities between the sexes or the experimental groups (Table IV). However, the GST activity correlated significantly with the PS dose ($r_s = 0.357$, $P < 0.05$).

Discussion

In this study, PS exposure caused multiple effects on endocrine parameters and metabolism of the field vole. In many cases, an induction of an enzyme or a change in the circulating hormone concentration was observed at 5 mg^{-1} kg^{-1} a day followed by a return to basal level at 50 mg^{-1} kg^{-1} a day. Of course, the relatively small number of animals could have affected the outcome. Yet, almost all of the statistically significant effects could be observed at the same PS dose, 5 mg^{-1} kg^{-1} a day, indicating that the observed differences were probably not due to chance. The wide range of parameters affected indicates that PS are not only

Table I. Food Intake, Body Mass, Length, and Relative and Absolute Organ Weights (Mean ± SE) of the Field Voles According to PS Dose

PS dose	0 mg kg^{-1} day^{-1}	5 mg kg^{-1} day^{-1}	50 mg kg^{-1} day^{-1}
Food intake (g)			
Males	54.3 ± 2.9	68.2 ± 3.5	63.2 ± 1.5[a]
Females	48.8 ± 1.2	47.2 ± 9.6	67.0 ± 8.7[a]
Body mass (g)			
Males[b]	23.3 ± 1.2	27.4 ± 1.3	22.2 ± 1.0
Females[b]	17.0 ± 1.4	17.7 ± 0.7	24.1 ± 3.6
Weight change (g)			
Males	−1.0 ± 0.5	−0.1 ± 1.2	+1.0 ± 0.3
Females	+0.3 ± 1.0	+1.4 ± 0.7	+0.4 ± 0.6
Length cm			
Males	9.5 ± 0.1	10.2 ± 0.1	9.8 ± 0.2
Females	9.0 ± 0.1	9.2 ± 0.2	9.9 ± 0.5
Liver weight (g)			
Males[b]	1.068 ± 0.094	1.077 ± 0.029	0.877 ± 0.052
Females[b]	0.800 ± 0.094	0.770 ± 0.029	0.948 ± 0.111
Liver weight (g body $mass^{-1}$ g × 100)			
Males	4.59 ± 0.29	3.95 ± 0.10[c]	4.00 ± 0.31[c]
Females	4.68 ± 0.24	4.35 ± 0.09[c]	4.05 ± 0.28[c]
Kidney weight (g)			
Males	0.232 ± 0.014	0.297 ± 0.021	0.228 ± 0.008
Females	0.211 ± 0.012	0.227 ± 0.012	0.271 ± 0.031
Kidney weight (g body $mass^{-1}$ g × 100)			
Males[b]	1.00 ± 0.04	1.08 ± 0.03	1.03 ± 0.04[c]
Females[b]	1.27 ± 0.09	1.28 ± 0.04	1.16 ± 0.06[c]
Testicular weight (g)	0.150 ± 0.02	0.227 ± 0.02	0.174 ± 0.02
Testicular weight (g body $mass^{-1}$ g × 1000)	6.59 ± 1.26	8.28 ± 0.78	7.83 ± 0.82

[a] Significant difference from the control groups (Mann-Whitney *U* test, $P < 0.05$).
[b] Significant difference between the sexes (*t* test, $P < 0.05$).
[c] These groups together differ significantly from the control groups together (Mann-Whitney *U* test, $P < 0.05$).

Table II. Activities of Liver (L) and Kidney (K) Enzymes of Carbohydrate and Lipid Metabolism and L or K Glycogen Content (Mean ± SE) of the Field Voles According to PS Dose

PS dose	0 mg kg^{-1} day^{-1}	5 mg kg^{-1} day^{-1}	50 mg kg^{-1} day^{-1}
L glycogen (μg mg^{-1})	10.44 ± 3.22	10.81 ± 1.63	9.92 ± 1.22
K glycogen (μg mg^{-1})	0.89 ± 0.09	0.91 ± 0.08	0.94 ± 0.07
L G6Pase (μg P mg^{-1} h^{-1})	85.7 ± 5.3[ab]	92.8 ± 4.1[b]	76.6 ± 5.4[a]
K G6Pase (μg P mg^{-1} h^{-1})	49.9 ± 4.8	43.1 ± 1.6	47.2 ± 3.9
L phosphorylase (μg P mg^{-1} h^{-1})	58.1 ± 4.5[a]	73.1 ± 3.1[b]	61.3 ± 1.9[a]
K phosphorylase (μg P mg^{-1} h^{-1})	8.5 ± 1.1[b]	6.4 ± 0.5[a]	5.9 ± 0.4[a]
L lipase esterase (μg 2-naphtol mg^{-1} h^{-1})	42.5 ± 3.8	43.6 ± 3.0	39.7 ± 3.0
K lipase esterase (μg 2-naphtol mg^{-1} h^{-1})	29.8 ± 1.0	28.6 ± 1.4	28.2 ± 2.6

Note. Values with dissimilar superscripts differ at $P < 0.05$ (one-way ANOVA).

natural mimics of estrogens but can also disrupt other physiological systems of the field vole.

Food intake of the voles increased due to PS exposure at 50 mg^{-1} kg^{-1} a day. This effect of PS has not been described previously. The effect could be mediated by the interference of PS to cholesterol absorption in the gut (6), causing increased appetite. However, body mass was unaffected. This may indicate increased metabolic rate, a hypothesis supported by the slight increase in circulating T_4 levels of the voles (Table III). The relative liver weights of the experimental animals decreased at all PS doses. This cannot be contributed to changes in the liver glycogen content, as it remained unchanged. However, the G6Pase activity did increase at 5 mg^{-1} kg^{-1} a day, but decreased again at 50 mg^{-1} kg^{-1} a day. The liver glycogen phosphorylase activity reflecting the rate of glycogenolysis (24) responded in the same way with an increase at 5 mg^{-1} kg^{-1} a day and a decrease at 50 mg^{-1} kg^{-1} a day. These changes indicate an enzyme induction in the liver at low doses and a return to basal level at higher doses. Thus, at 5 mg^{-1} kg^{-1} a day, the rate of glucose turnover was the highest. However, previous findings on the effects of PS on the physiology of the European polecat (*Mustela putorius*), a carnivore, showed a significant increase in the liver glycogen content and a decrease in the liver lipase esterase activity due to PS (25). In this study, the effects targeted on different enzymes emphasized the considerable interspecies differences in the reactions to PS.

Table III. Plasma Hormone Concentrations (Mean ± SE) of the Field Voles According to PS Dose

PS dose	0 mg kg^{-1} day^{-1}	5 mg kg^{-1} day^{-1}	50 mg kg^{-1} day^{-1}
Estradiol (nmol l^{-1})			
Males	0.13 ± 0.04^{a}	3.76 ± 1.74^{b}	0.09 ± 0.02^{a}
Females	0.30 ± 0.23^{a}	0.73 ± 0.32^{ab}	0.14 ± 0.03^{a}
Testosterone (nmol l^{-1})			
Males	0.13 ± 0.04^{a}	0.85 ± 0.68^{b}	0.25 ± 0.11^{ab}
Females	0.09 ± 0.01^{a}	0.11 ± 0.02^{a}	0.09 ± 0.01^{a}
LH (mIU ml^{-1}; pooled)	1.671	0.181	1.186
T_4 (nmol l^{-1})	22.7 ± 1.8	26.0 ± 2.8	24.8 ± 1.5
TSH (mIU ml^{-1}; pooled)	0.443	BD	BD
Leptin (ng ml^{-1}; pooled)	4.10	4.60	4.30
Ghrelin (ng ml^{-1}; pooled)	0.3	0.2	0.3

Note. Values with dissimilar superscripts differ at $P < 0.05$ (two-way ANOVA). (BD, below the detection limit).

Table IV. Activities of Liver Biotransformation Enzymes (Mean ± SE) of the Field Voles According to PS Dose

PS dose	0 mg kg^{-1} day^{-1}	5 mg kg^{-1} day^{-1}	50 mg kg^{-1} day^{-1}
EROD (pmol min^{-1} mg^{-1} protein)	1848 ± 135	1655 ± 224	1497 ± 179
UDPGT (pmol min^{-1} mg^{-1} protein)	1160 ± 140	980 ± 80	1100 ± 70
GST (μmol min^{-1} mg^{-1} protein)	4.36 ± 0.36	4.75 ± 0.22	5.52 ± 0.49

The relative kidney weight, on the other hand, decreased at 50 mg^{-1} kg^{-1}. At the same time, the kidney glycogen phosphorylase activity decreased at all PS doses, indicating decreased glycogen degradation. Thus, the changes in the kidney glycogen content do not by themselves reduce the relative kidney weight. As this enzyme in the kidney provides energy for the organ itself (24), it is possible that PS also have direct effects on intermediary metabolism in the kidney–a phenomenon that should to be looked into in the future.

The observed increase in the plasma estradiol concentrations at 5 mg^{-1} kg^{-1} a day was the most obvious change in the endocrine parameters measured, especially in males. Also in this case, the effect could be observed at a low dose. At the same time, the LH concentration measured from pooled plasma dropped at 5 mg^{-1} kg^{-1} a day. This seems likely to be due to negative feedback exerted by estradiol on LH secretion in the pituitary. The increase in the plasma estradiol concentrations also conforms to the results of previous studies on the polecat (25). A similar trend could also be observed in the plasma testosterone levels, with the highest mean concentration at 5 mg^{-1} kg^{-1} a day. Especially interesting was the fact that the circulating estradiol and testosterone levels were the highest in males exposed to PS at 5 mg^{-1} kg^{-1}. PS accumulate in the liver, adrenals, and gonads of rats (4), and they can be used as precursors of cortisol and sex steroid biosynthesis (3, 4). It seems possible that the increased estradiol and testosterone levels of male voles at 5 mg^{-1} kg^{-1} could be caused by PS being used in steroid hormone synthesis. The mean testicular weight was also the highest at 5 mg^{-1} kg^{-1}. This fits to the increase of the circulating testosterone levels in the same experimental group. The effects observed on the field vole differ from previous results obtained in various fish species with lowered sex steroid levels due to PS treatment (13).

Apart from estradiol and testosterone, no clear sex-specific effects could be seen in the endocrine parameters of the voles. The observed increase in sex steroid levels can be due to increased synthesis from PS precursors (3, 4), but at present, there is no explanation as to why this effect would be more pronounced in males. However, it is known that the female of the genus is an induced ovulator. Ovulation and LH surge occur in females only due to behavioral triggers, pairing, and copulation (26, 27). Unpaired females have erratic estrous cycles, but their reproductive hormone levels are significantly lower. As the voles in this experiment were housed singly, it is possible that the lack of behavioral triggers dampened the increase of reproductive hormone concentrations in females.

In this study, no effects of PS could be observed on the liver biotransformation enzyme activities. This is also in concert with previous results on the polecat, with no obvious induction of the biotransformation apparatus (25). Thus, PS do not seem to be recognized as foreign compounds, and due to this, they can exert their diverse effects unhindered. The only exception was the liver GST activity, which correlated positively with the PS dose. This suggests some activation of the phase II biotransformation. This is very different from the effects of chemically derived endocrine disruptors. Bisphenol A (BPA), for instance, inhibits the male-specific EROD isoforms of rats (28). BPA also decreases the EROD and GST activities of the field vole (16). Thus, it is impossible to use biotransformation activity as a biomarker for endocrine disruption caused by PS in nature. However, the elevated estradiol levels of both male and female prebreeding field voles could be used as indicators of increased PS exposure in land ecosystems.

In summary, PS exposure caused multiple effects on the endocrinology and physiology of the field vole. A typical effect was an induction of enzyme activity or hormone

secretion at low levels and a return to the basal levels of control animals at a higher dose. This effect is puzzling, but similar phenomena have been encountered in the rainbow trout due to PS exposure with increased EROD activity at low or high doses, but with suppression of EROD at intermediate doses. As most of the effects were the most pronounced at 5 mg^{-1} kg^{-1} a day, it is also possible that at higher doses, other mechanisms beside the liver biotransformation enzymes counteract the effects of PS successfully. However, 5 mg^{-1} kg^{-1} a day is the recommended dose of β-sitosterol in various health products containing PS. In this regard, part of the general population consumes PS at a rate high enough to cause some of the effects described above. Of course, the observed effects per se were not beneficial or harmful. However, they were quite distinct even after a relatively short exposure. As wild mammals ingest continuously other PS and foreign substances, there can also be complex interactions neglected in this study. A thorough risk assessment with chronic exposure on the effects of PS should be taken into consideration.

We thank Jyrki Pusenius for acquiring the original stock of field voles. Leena Koponen kindly took good care of our critters. Mr. Ulf Hotanen provided us with the PS. The technical assistance of Anita Kervinen, Eeva-Liisa Palkispää, and Riitta Venäläinen is highly appreciated.

1. Drexel H, Breier C, Lisch H-J, Sailer S. Lowering plasma cholesterol with β-sitosterol and diet. Lancet **23:**1157, 1981.
2. Miettinen TA, Strandberg TE, Gylling H. Noncholesterol sterols and cholesterol lowering by long-term simvastatin treatment in coronary patients. Relation to basal serum cholesterol. Arterioscler Thromb Vasc Biol **20:**1340–1346, 2000.
3. Moghadasian MH. Pharmacological properties of plant sterols. In vivo and in vitro observations. Life Sci **67:**605–615, 2000.
4. Sanders DJ, Minter HJ, Howes D, Hepburn PA. The safety evaluation of phytosterol esters. Part 6. The comparative absorption and tissue distribution of phytosterols in the rat. Food Chem Toxicol **38:**485–491, 2000.
5. Jones PJ, Raeini-Sarjaz M, Ntanios FY, Vanstone CA, Feng JY, Parsons WE. Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. J Lipid Res **41:**697–705, 2000.
6. Mattson FH, Grundy SM, Crouse J. Optimizing the effect of plant sterols on cholesterol absorption in man. Am J Clin Nutr **35:**697–700, 1982.
7. Hallikainen MA, Sarkkinen ES, Gylling H, Erkkilä AT, Uusitupa MIJ. Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet. Eur J Clin Nutr **54:**715–725, 2000.
8. Awad AB, Fink CS. Phytosterols as anticancer dietary components: evidence and mechanism of action. J Nutr **130:**2127–2130, 2000.
9. Awad AB, Gan Y, Fink CS. Effect of β-sitosterol, a plant sterol, on growth, protein phosphatase 2A and phospholipase D in LNCaP cells. Nutr Cancer **36:**74–78, 2000.
10. Raicht RF, Cohen LI, Fazzini EP, Sarwall AN, Takahashi M. Protective effect of plant sterols against chemically induced colon tumors in rats. Cancer Res **40:**403–405, 1980.
11. Van der Kraak GJ, Munkittrick KR, McMaster ME, Portt CB, Chang JP. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. Toxicol Appl Pharmacol **115:**224–233, 1992.
12. Mellanen P, Petänen T, Lehtimäki J, Mäkelä S, Bylynd G, Holmbom B, Mannila E, Oikkari A, Santti R. Wood-derived estrogens: studies in vitro with breast cancer cell lines and in vivo in trout. Toxicol Appl Pharmacol **136:**381–388, 1996.
13. MacLatchy DL, Van der Kraak G. The phytoestrogen β-sitosterol alters the reproductive endocrine status of goldfish. Toxicol Appl Pharmacol **134:**305–312, 1995.
14. Krapp F, Niethammer J. *Microtus agrestis* (Linnaeus, 1761): Erdmaus. In: Niethammer J, Krapp F, Eds. Handbuch der Säugetiere Europas. Rodentia II (Cricetidae, Arvicolidae, Zapodiae, Spalacidae, Hystricidae, Capromyidae). Wiesbaden: Akademische Verlagsgesellschaft Band 2/I, pp349–373, 1982.
15. Myllymäki A. Demographic mechanisms in the fluctuating populations of the field vole, *Microtus agrestis*. Oikos **29:**553–569, 1977.
16. Nieminen P, Lindström-Seppä P, Mustonen A-M, Mussalo-Rauhamaa H, Kukkonen JVK. Bisphenol A affects endocrine physiology and biotransformation enzyme activities of the field vole (*Microtus agrestis*). Gen Comp Endocr **126:**183–189, 2002.
17. Hers HG, van Hoof F. Enzymes of glycogen degradation in biopsy material. In: Colowick S, Kaplan NO, Eds. Methods in Enzymology. New York: Academic Press, Vol. **VIII:**pp525–532, 1966.
18. Seligman AM, Nachlas MM. Lipase. In: Bermayer HU, Ed. Methoden der Enzymatischen Analyse. Weinheim: Verlag Chemie GmbH, pp776–778, 1962.
19. Lo S, Russel J, Taylor AW. Determination of glycogen in small tissue samples. J Appl Physiol **28:**234–236, 1970.
20. Burke MD, Mayer RT. Ethoxyresorufin: direct fluorimetric assay of microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab Dispos **2:**583–588, 1974.
21. Hänninen O. On the metabolic regulation in the glucuronide acid pathway in the rat tissues. Ann Acad Sci Fenn **A2:**1–96, 1968.
22. Habig WH, Pabst MJ, Jakoby WB. Glutathione *S*-transferase. The first enzymatic step in mercapturic acid formation. J Biol Chem **249:**7130–7139, 1974.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:**248–254, 1976.
24. Harris RA. Carbohydrate metabolism I: major metabolic pathways and their control. In: Devlin TM, Ed. Textbook of Biochemistry with Clinical Correlations. Singapore: John Wiley & Sons, pp261–328, 1986.
25. Nieminen P, Mustonen A-M, Lindström-Seppä P, Asikainen J, Mussalo-Rauhamaa H, Kukkonen JVK. Phytosterols act as endocrine and metabolic disruptors in the European polecat (*Mustela putorius*). Toxicol Appl Pharmacol **178:**22–28, 2002.
26. Chitty H, Austin CR. Environmental modification of oestrus in the vole. Nature **179:**592–593, 1957.
27. Breed WG. Ovulation in the genus *Microtus*. Nature **241:**826, 1967.
28. Hanioka N, Jinno H, Nishimura T, Ando M. Suppression of male-specific cytochrome P450 isoforms by bisphenol A in rat liver. Arch Toxicol **72:**387–394, 1998.