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Effects of phytoestrogens on the reproduction and weight regulation of mammals

by
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The aim of this study was to investigate the effects of dietary phytoestrogens, genistein and phytosterols (mainly β-sitosterol), at doses reflecting their intake by humans, on the reproduction, post-natal development and weight regulation of mink (*Mustela vison*) and mice (*Mus musculus*). General parameters and concentrations of plasma hormones and lipids were monitored across generations (F_0-F_4 in mice exposed to phytosterols and F_0-F_1 in mink and mice exposed to genistein and β -sitosterol).

The onset of the testosterone peak before mating was accelerated in phytoestrogen-exposed male mink and β-sitosterol females had slightly larger litters than the controls. Phytoestrogen-exposed mink kits were gain less body weight than the controls, their plasma thyroxine levels decreased and there were changes in the weights of their reproductive organs. Plasma leptin levels increased in adult male mice exposed to phytosterols, while genistein treatment reduced the plasma ghrelin levels in adult females and increased the relative weights of the prostate gland and seminal vesicles in male pups.

The results partly confirm earlier findings that exposure to phytoestrogens in the early life stages of experimental animals produces the most noticeable effects. Being carnivorous, the mink seem to be more sensitive to phytoestrogen exposure than mice, which are omnivorous and have become adapted to phytoestrogens via their diet. Many of the observed changes can be taken as signs of endocrine disruption, although phytoestrogen exposure had no adverse effects on reproduction at the studied exposure levels.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by Roman numerals (I-V).

I Ryökkynen, A., Nieminen, P., Mustonen, A-M., Pyykönen, T., Asikainen, J., Hänninen, S., Mononen, J. and Kukkonen, J.V.K. 2005: Phytoestrogens alter the reproductive organ development in the mink (*Mustela vison*). Toxicol Appl Pharmacol 202, 132-139.

II Ryökkynen, A., Käyhkö, U-R., Mustonen, A-M., Kukkonen, J.V.K. and Nieminen, P. 2005: Multigenerational exposure to phytosterols in the mouse. Reprod Toxicol 19, 535-540.

III Ryökkynen, A., Kukkonen, J.V.K. and Nieminen, P. 2005: Effects of dietary genistein on mouse reproduction, weight-regulation and postnatal development. Anim Reprod Sci 93, 337-348.

IV Ryökkynen, A., Mustonen, A-M., Pyykönen, T., and Nieminen, P. 2006: Endocrine and metabolic alterations in the mink (*Mustela vison*) due to chronic phytoestrogen exposure. Chemosphere, in press. Published online, doi:10.1016/j.chemosphere.2005.12.037.

V Ryökkynen, A., Mustonen, A-M., Kukkonen, J.V.K. and Nieminen, P. 200x: Dietary phytosterol exposure increases plasma leptin levels of the male mouse in a multigenerational study. Submitted.

I participated in the design of studies (I-V) and was mainly responsible for the sampling, the data collection, the data analysis and the preparation of the manuscripts. The processing of the articles was carried out in collaboration with the co-authors.

1 INTRODUCTION

Phytoestrogens are compounds that occur naturally in plants. They are structurally and/or functionally similar to mammalian estradiol and they have estrogenic and/or anti-estrogenic effects on animals. Phytoestrogens are released to the environment from paper and pulp mill effluents (Cook et al., 1997; Kiparissis et al., 2001) and sewage waste waters (Spengler et al., 2001; Pawlowski et al., 2003). Some phytoestrogens have been categorized as endocrine disruptors, i.e. exogenous substances that alter the function(s) of the endocrine system and consequently have adverse effects on the health of the intact organism, its progeny, or its (sub)populations (Commission of the European communities, 1999). They mimic or block hormones and disrupt normal endocrine functions by altering normal hormone levels, inhibiting or stimulating the production of hormones or changing the way hormones circulate in the body. This study is concerned with genistein and β-sitosterol, which some sources classify as natural environmental estrogens (Arukwe, 2001) or natural endocrine-active agents (Safe et al., 1997). The Scientific Committee on food of the European Commission (phytosterols) and the U.S. Food and Drug Administration (phytosterols and genistein) have sanctioned the use of phytoestrogens in foods (SCF, 2002; SCF, 2003a; SCF, 2003b; U.S. Food and Drug Administration, 2005), with some guidelines for their use (Lichtenstein and Deckelbaum, 2001; Byrne, 2004). In the case of genistein, no guidelines have been determined (Setchell, 2000).

Scientific interest in phytoestrogens increased in the 1940's when sheep (*Ovis aries*, Linneaus 1758) that had been eating subterranean clover (*Trifolium subterraneum*, L.), which contains high concentrations of phytoestrogens, were found to have reproductive dysfunctions (cystic ovaries, irreversible endometriosis and a failure to conceive; Bennetts et al., 1946). Earlier experiments had shown that dietary sitosterol reduced the levels of liver cholesterol in mice (*Mus musculus*, Linnaeus 1758; Sperry and Bergmann, 1937) and later dietary soy sterols were found to lower the levels of plasma and liver cholesterol in white leghorn chicks (*Gallus gallus domesticus*, Linnaeus 1758; Peterson, 1951). Since then, numerous studies have demonstrated that dietary plant sterols reduce plasma cholesterol levels in human beings (*Homo sapiens*, Linnaeus 1758; Pollak, 1953a; Best et al., 1954; Farquhar et al., 1956; Best and Duncan, 1956), dogs (*Canis lupus familiaris*, Linnaeaus 1758; Shipley et al., 1958), rabbits (*Oryctolagus cuniculus*, Linnaeaus 1758; Pollak, 1953b; Shipley et al., 1958) and rats (*Rattus norvegicus*, Berkenhout 1769; Shipley et al., 1958; Best and Duncan, 1956).

Phytoestrogens have been part of traditional diet in Asia for millennia without showing any adverse effects. On the contrary, the ingestion of phytoestrogens has been associated with many benefits. For example, it has been suggested that they offer potential protection against cardiovascular disease and various kinds of cancers, such as breast, colon, and prostate cancer (Setchell and Cassidy, 1998; Messina, 2003). Genistein might be effective in reducing menopausal symptoms (Munro et al., 2003) and it may provide an alternative treatment for osteoporosis (Anderson and Garner, 1997; Setchell and Lydeking-Olsen, 2003), while β-sitosterol might render benign prostate hyperplasia asymptomatic (Berges et al., 1995) and protect against colon, breast and prostate cancer, as demonstrated in experimental and epidemiological studies (Awad and Fink, 2000; McCann et al., 2005). It has also been observed *in vitro* that β-sitosterol can inhibit the growth of human breast cancer cells (MDA-MB-231; Awad et al., 2003) and mouse colonic epithelial cells (Janezic and Rao, 1992). The only side-effect observed to date is that

phytosterols can reduce the absorption of some fat-soluble vitamins by 15% (α-carotene, β-carotene, lycopene; SCF, 2002; Kritchevsky and Chen, 2005; Plat and Mensink, 2005).

Some adverse effects of phytoestrogens have been observed in experimental animals. For example, dietary isoflavones have caused infertility in male mice (East, 1955) and rats (Nagao et al., 2001). It has been also shown that exposure to β-sitosterol and genistein caused adverse effects on reproductive organ weights in mice (Strauss et al., 1998; Wisniewski et al., 2005) and rats (Malini and Vanithakumari, 1991; Nagao et al., 2001). Exposure to phytosterols and genistein reduced plasma testosterone levels in rats (Awad et al., 1998; Wisniewski et al., 2005) and similar reductions of the plasma estradiol and progesterone levels have been noticed in female rats exposed to genistein (Awoniyi et al., 1998). On the other hand, it has been shown that phytosterol exposure increased plasma testosterone levels in the European polecat (*Mustela putorius*, Linnaeus 1758; Nieminen et al., 2003b) and in the field vole (*Microtus agrestis*; Linnaeus 1761; Nieminen et al., 2003c).

1.1 STRUCTURE OF PHYTOESTROGENS

1.1.1 Phytosterols

More than 200 phytosterols have been classified. They belong to the family of triterpenes, which are important components of plant membranes (for reviews see, Moreau et al., 2003; Schaller, 2003). They stabilize phospholipid bilayers in plant cell membranes in the same way that cholesterol does in animal cell membranes. They are similar in structure to animal cholesterol but differ in the side chain at C-24 and/or in the position and configuration of unsaturated double bonds and the optical rotation at chiral carbons (Fig. 1). Most phytosterols contain 28 or 29 carbon atoms. Phytostanols are a fully saturated subgroup of phytosterols which contain no double bonds. They are less abundant in nature than sterols.

The main dietary sources of phytosterols are vegetable oils, seeds, nuts and cereals (Frische and Steinhart, 1999; Piiroinen et al., 2000; Moreau et al., 2003; Valsta et al., 2004; Phillips et al., 2005). They are also incorporated into several functional foods (margarine spreads, milk, salad dressings, yoghurt, cheese, snack products, sausages; SCF, 2003a) and sold as natural products (tablets, powders) that may be beneficial to health. The principal phytosterols in foods are β-sitosterol, campesterol and stigmasterol (Phillips et al., 2005).

1.1.2 Isoflavones

Isoflavones are different from the other flavonoid classes that belong to polyphenols, because they contain a rearranged C15 skeleton based on 3-phenylchroman (Williams and Harborne, 1989; Manach et al., 2004). Genistein belongs to the best-known group of isoflavones, aglycones. There are 234 different aglycones, only a few of which have estrogenic activities similar to those of genistein. The basic structure of genistein is similar to that of naturally occurring steroidal estrogens like estradiol (Fig. 2). It has been known since 1931 that genistein is contained in soybeans (Walz, 1931). It has also been detected in many other food items, such as legumes, grains, vegetables, herbs and fruits (Coward et al., 1993; Frische and Steinhart, 1999; Liggins et al., 2000; de Kleijn et al., 2001; Hui et al., 2001; Valsta et al., 2003). Isoflavones, mainly genistein and daidzein, are found in many natural products that are marketed as promoting health and used to treat various diseases such as asthma, cancer, menopause, osteoporosis, atherosclerosis (Setchell et al., 2001), as well as in many commercial pet foods (Brown and Setchell, 2001) and soy-based infant formulas (Setchell et al., 1997; Setchell et al., 1998).

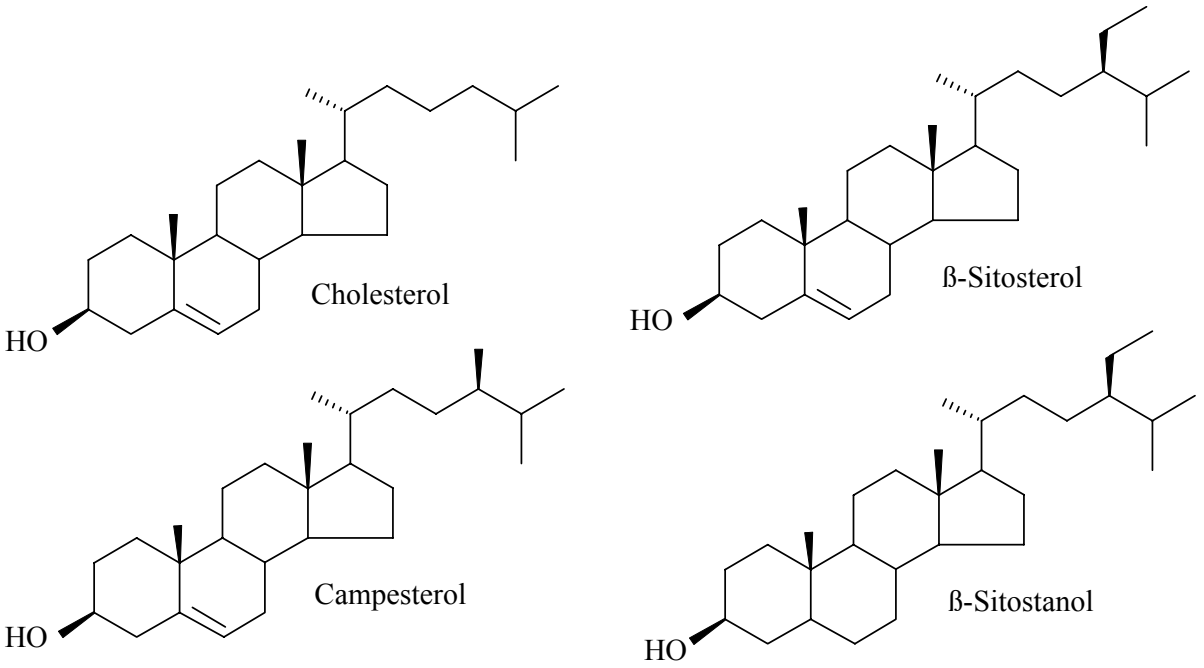

Figure 1. The structures of **cholesterol** (MW=386.67; $C_{27}H_{46}O$; CAS 57-88-5) = Cholest-5-en-3beta-ol, 3β-hydroxy-5-cholestene) and the phytosterols used in this study. **β-sitosterol** (MW=414.71; $C_{29}H_{50}O$; CAS 83-46-5) = α-Dihydrofucosterol, 22,23-Dihydrostigmasterol, 22,23-Dihydrostigmasterol, 24β-Ethylcholesterol, 5-Stigmasten-3β-ol. **Campesterol** (MW=400.68; $C_{28}H_{48}O$; CAS 474-62-4) = 24α-Methyl-5-cholesten-3β-ol, 24α-Ergost-5-en-3β-ol). **β-sitostanol** (MW=416.40; $C_{29}H_{52}O$; CAS 19466-47-8) = β-Sistostanol, 24α-Ethyl-5α-cholestan-3β-ol, 24α-Ethyl-5α-cholestan-3β-ol, Dihydro-β-sitosterol, Stigmastan-3β-ol.

1.2 WHAT HAPPENS TO PHYTOESTROGENS IN THE BODY

Since phytoestrogens are not synthesized in animals, those present in animal plasma and tissue are entirely of dietary or maternal origin (Holmes and Phillips, 1999; von Bergmann et al., 2005). It has been shown that phytoestrogens differ greatly in their absorption, distribution, metabolism and excretion (de Jong et al., 2003; Rowland et al., 2003). In addition, biological responses to phytoestrogen exposure are affected by many other factors, such as the species, age, gender, diet, dose, and the route of administration. Many studies use exposure routes, such as subcutaneous administration, whereby the studied substance bypasses gut microflora and hepatic first pass metabolism. This may have a significant impact on its biological potency, because the natural exposure route of phytoestrogens is via placenta and lactation in offspring or via peroral ingestion in adults. It has been assumed that animals are able to metabolize and excrete phytoestrogens and consequently they would not accumulate in tissues under normal conditions.

1.2.1 Absorption

The principal site for the absorption of phytoestrogens is the small intestine. Many studies have investigated the rate and extent of absorption of phytoestrogens by measuring their plasma, urinary, faecal and tissue concentrations (Ikeda and Sugano, 1978; Mellies et al., 1978; Adlercreutz et al., 1991; Chang et al., 2000; Sanders et al., 2000; Batta et al., 2005). It is well-known that phytosterols are poorly absorbed from the gastro-intestinal tract as compared to cholesterol (Schönheimer et al., 1930; Sperry and Bergmann, 1937; Dunham et al., 1959; Swell et al., 1959; Mattson et al.,

1982; Sanders et al., 2000; Sehayek, 2003) or genistein (Rowland et al., 2003), which are absorbed readily from the intestine.

The absorption of phytosterols ranges from little (~8%) to almost none, depending on the gender, species, individual, and the methods used (Schönheimer et al., 1930; Boorman and Fisher, 1966; Borgström, 1968; Sylvén and Borgström, 1969; Salen et al., 1970; Sehayek, 2003). The absorption of different phytosterols also varies (Subbiah, 1973). For example, the absorption rate of campesterol (C_{28}) is higher than that of β-sitosterol (C_{29}). The uptake of phytosterols from the intestine is rapid (Igel et al., 2003), but the plasma levels remain low with dietary exposure. In very rare cases, such as those due to the genetic disorder, sitosterolemia, the plasma phytosterol concentrations can be extremely high (Bhattacharyya and Connor, 1974; Sehayek, 2003). It has been suggested that the absorption rate of phytosterols is affected by several factors (de Jong et al., 2003; Sehayek, 2003), including micellar solubility (Slota et al., 1983; Ikeda et al., 1988a), slow transfer of sitosterol to the intracellular space and mucosal esterification (Ikeda et al., 1988b).

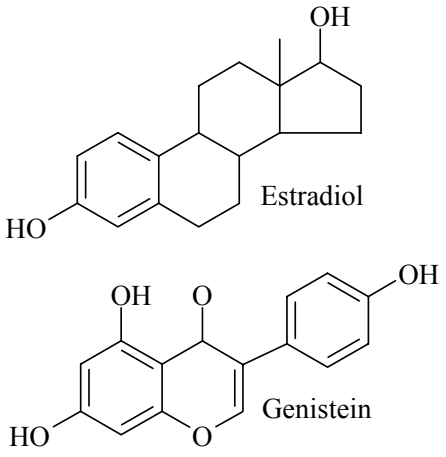

Figure 2. The structures of **estradiol** (MW=272.39; $C_{18}H_{24}O_2$; CAS 50-28-2) = 17-beta-estradiol, 1,3,5-estratriene-3,17-beta-diol and **genistein** (MW=270.23; $C_{15}H_{10}O_5$; CAS 446-72-0) = 4',5,7-Trihydroxyisoflavone, 5,7-Dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one.

Genistein, on the other hand, is absorbed easily and rapidly by passive diffusion from the intestine (Sfakianos et al., 1997; Crespy et al., 2003). One important element affecting the absorption and biological activity of genistein is the gender and age of the exposed individual. Another is intestinal microflora, since after ingestion all isoflavones are hydrolyzed by intestinal glucosidases (Setchell et al., 1984; Setchell and Cassidy, 1998, Rowland et al., 2003).

1.2.2 Distribution

Absorbed phytoestrogens and/or their biotransformation products are distributed very effectively via circulation throughout the body. It has been shown that very small amounts of phytosterols can accumulate in the blood, the brain, the lungs, the heart, the liver, the kidneys, the ovaries, the uterus, the testes, the adrenals, the stomach, the small intestine and the large intestine (Sanders et al., 2000), while genistein seems to accumulate dose-dependently in the thyroid gland (Chang and Doerge, 2000), the liver, the brain, the mammary glands, the ovaries, the prostate, the testes and the uterus (Chang et al., 2000). It has been observed that both genistein and β-sitosterol can cross the placenta (Mellies et al., 1976; Adlercreutz et al., 1999; Doerge et al., 2001; Todaka et al., 2005) and that they can be excreted in the milk in humans (Mellies et al., 1978; Setchell et al., 1997) and rats (Fritz et al., 1998). Lactation has been shown to raise the concentrations of plasma phytoestrogens in the offspring of rats exposed to genistein (Fritz et al., 1998).

1.2.3 Metabolism

Metabolism is the process by which an ingested phytoestrogen is structurally modified in the body by different enzymatic or non-enzymatic reactions. In the intestine, phytosterols compete with cholesterol and interact with cholesterol absorption (Sehayek, 2003). Some phytosterols may be esterified in the liver (Subbiah, 1973), secreted into the bile acids unchanged

(Aringer, 1978) or partly secreted into plasma, mostly as HDL cholesterol (Sehayek, 2003), especially in rats (Sugano et al., 1978; Robins and Fasulo, 1997). In contrast to other plasma sterols, such as cholesterol, plant sterols have little or no capacity to be metabolised into bile acids in the liver of a healthy human being (Boberg et al., 1990). It is surmised that metabolites of phytosterols are of little or no importance to the well being of animals (Kuksis, 2001). However, it has been suggested that β-sitosterol may be converted to cholesterol in guinea pigs (*Cavia porcellus*, Pallas 1766; Werbin et al., 1960) and tobacco hornworms (*Manduca sexta*, Linnaeus 1763; Svoboda et al., 1967) and thus, may be able to act as a precursor for sex steroids, at least in some animal species.

It has been demonstrated that aglycones can undergo fermentation by intestinal bacteria since, in humans, genistein can be metabolized to dihydrogenistein and then to 6-hydroxy-0-desmethylangolensin (Rowland et al., 2003). In rats, six other genistein metabolites have been found: four monohydroxylated (5,6,7,4'-tetrahydroxy-isoflavone, 5,7,3'4'-tetrahydroxyisoflavone, 5,7,8,4'-tetrahydroxyisoflavone and 2,5,7,4'-tetrahydroxyisoflavone) and two dihydroxylated (5,6,7,3'4'-pentahydro-xyisoflavone and 5,7,8,3'4'-pentahydro-xyisoflavone) metabolites; (Kulling et al., 2000). Some, such as 4-hydroxyphenyl-2-propionic (Coldham and Sauer, 2000) or *p*-ethylphenol (King, 1998; Yasuda et al., 2001), have not been found in humans, indicating that there are species-specific differences in the metabolism of genistein. Genistein is absorbed from the large and small intestines and is eventually transported to the liver where it partly undergoes conjugation with glucuronate and sulfate via hepatic phase II enzymes (glutathione S-transferase, UDP-glucurono-syltransferases, quinone reductase and sul-fotransferases; Eaton et al., 1996; Yannai et al., 1998; Dingley et al., 2003). Its metabolites are excreted in the bile as 7-O-β-glucuronide conjugate (Sfakianos et al., 1997). Genistein conjugates may be deconjugated to release genistein, which may then be reabsorbed via the entero-hepatic circulation. However, it has been shown in mammals that phytosterols do not activate the biotransformation of xeno-biotics *in vivo* (Nieminen et al., 2003a; Nieminen et al., 2003b; Nieminen et al., 2003c), in contrast to genistein (Eaton et al., 1996; Yannai et al., 1998; Dingley et al., 2003).

1.2.4 Excretion

Ingested phytoestrogens and their metabolites are excreted mostly via urine and faeces but small concentrations have also been measured in milk (Mellies et al., 1978; Franke and Custer, 1996; Setchell et al., 1997; Fritz et al., 1998), semen (Tham et al., 1998), amniotic fluid (Adlercreutz et al., 1999), saliva and prostatic fluids (Finlay et al., 1991). The excretion rate is affected by many factors and there is great individual variability. In rats, the major route of excretion for phytosterols is the faeces (>75%, Sanders et al., 2000): one third of ingested genistein is excreted in faeces and the rest via urine (King, 1998; Coldham and Sauer, 2000). In human beings, the main excretion route is via the kidney (Rowland et al., 2003). It has been shown that most ingested genistein is excreted as metabolites in urine, and the rest in unconjugated form in urine (approximately 12-30%) and faeces (about 4%, King, 1998; Rowland et al., 2003). It has been observed that after prolonged exposure to genistein the half-life of excretion becomes shorter in females but longer in males (Lu and Anderson, 1998).

1.3 AIMS OF THE STUDY

Although knowledge of the effects of phytoestrogens has increased recently, there have been only a few studies of long-term exposure over generations. The aim of this research was to examine the effects of two phytoestrogens (genistein and β-sitosterol), at doses reflecting intake by humans, on the

reproduction, postnatal development and weight regulation of mice (II, III, V) and mink (I, IV). General parameters, hormones, lipoprotein fractions, food consumption, body-weight changes and metabolic enzymes were measured to get a broad view of the possible effects of exposure to phytoestrogens.

2 MATERIALS AND METHODS

2.1 ANIMALS AND EXPERIMENTAL PROTOCOLS

Two animal species were chosen for these studies. The American mink (*Mustela vison*, Schreber 1777) is a medium-sized, seasonally breeding, semi-aquatic, mustelid carnivore (Stubbe, 1993). It is a top predator and it is widely used as a highly sensitive indicator species in studies of environmental contaminant exposure and ecosystem health (Smits et al., 1995; Harding et al., 1999; Beckett et al., 2002). The common house mouse is a small omnivore which is a member of the family *Muridae*. The mouse is widely used in laboratory experiments.

Sixty farm-bred wild-type mink were randomly divided into three groups: 1) control, 2) β-sitosterol and 3) genistein (I, IV). The animals were paired and housed in standard wire cages (85 x 31 x 38 cm) with wooden nest boxes (27 x 31 x 38 cm). The cages were raised above ground in a barn at the Juankoski Research Station of the University of Kuopio (63ºN; 28ºE). The animals were kept in natural photoperiod and temperature. They had free access to water and food was offered twice a day. Twenty male mice and twenty female mice (NIH/S) were randomly divided into two experimental groups, one receiving control feed and the other phytosterol mixture (II, V). A further twenty pairs of male and female mice were selected to receive a phytoestrogen-free diet, ten pairs with genistein and ten without (III). The main features of the experimental protocols are described in Figure 3. More detailed explanations can be found in the materials and methods sections of the original publications (I-V). All procedures were approved by the Animals' Care and Use Committee of the University of Joensuu.

2.2 CHEMICALS

Genistein (99% purity) at 8 mg/kg body weight (BW)/day was used in the mice and the mink studies (LClaboratories, Woburn, MA, USA; I, III, IV), β-sitosterol at 50 mg/kg BW/day in mink studies (β-Sitosterol S-5753, Sigma-Aldrich CO, St.Louis, MO, USA; I, IV) and phytosterol mixture at a dose of 5 mg/kg BW/day in the mice studies (Ultrasitosterol®, UPM Kymmene Corporation, Kaukas, Lappeenranta, Finland; β-sitosterol 75.7%, β-sitostanol 13%, campesterol 9%, artenols 0.9%; II, V). The genistein dose (I, III, IV) was based on the amount of genistein consumed by infants receiving soy formulas (Setchell et al., 1997). The β-sitosterol dose (I, IV) was based on those used in dose-response studies of the European polecat (Nieminen et al., 2003b). It was also close to the dose recommended for the treatment of hypercholesterolemia (1-3 g/day = 14-43 mg/kg BW/day, Moreau et al., 2003; SCF, 2003a; SCF, 2003b; Thompson and Grundy, 2005). The phytosterol mixture was similar to the lowest dose found to cause changes in the plasma sex hormone levels of field voles in a dose-response study (Nieminen et al., 2003c) and close to the amount ingested daily by humans consuming a vegetarian diet (Cerquira et al., 1979; Nair et al., 1984).

2.3 BIOCHEMICAL ANALYSES

The amounts of plasma hormones (dihydrotestosterone (DHT), estradiol, ghrelin, leptin, progesterone, testosterone, thyroxine (T_4), tri-iodothyronine (T_3)) were determined using radioimmunoassay (RIA) methods, except in study II where the plasma estradiol concentrations of the mice were determined using the Enzyme-Linked Immunosorbent Assay (ELISA) method. The measurements were carried out according to the manufacturers' protocols.

The actual RIA measurements were made with a gamma counter (Wizard 1480, Wallac, Turku, Finland) and the ELISA measurements with a Microplate Reader (Multiskan Ascent®, Labsystems, Helsinki, Finland). The plasma lipids (total cholesterol, high and low density lipoprotein cholesterol, triglycerides), glucose and total protein concentrations were measured spectrofotometrically (Technicon RA-XT™ System, Technicon Ltd., Dublin, Ireland), using commercial reagents (Randox Laboratories, Crumlin, Co.Antrim, UK). Details of all measurements can be found in the original publications (I-V).

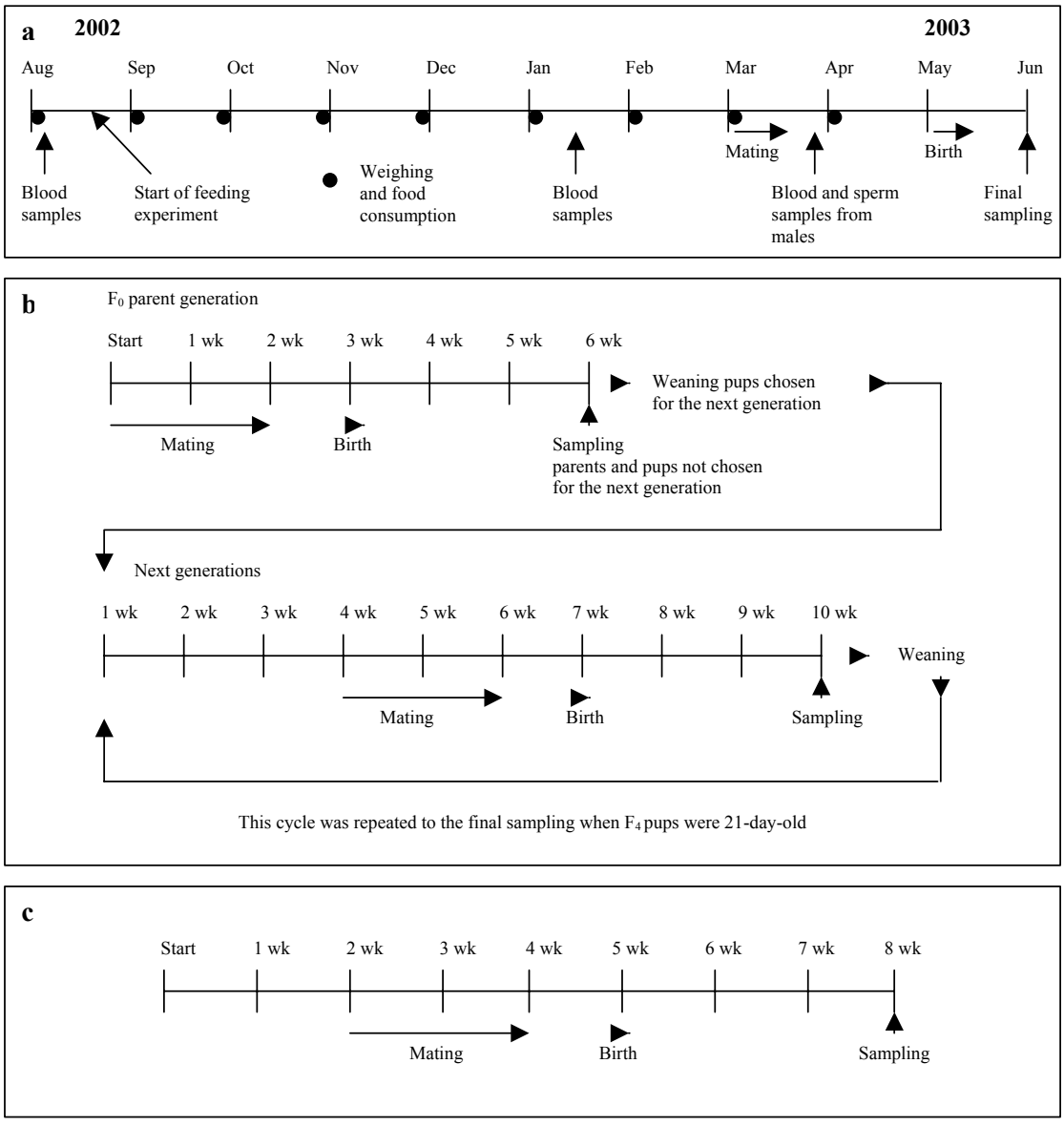

Figure 3. The experimental protocols of the studies with mink (a; I, IV) and mice (b; II, V; phytosterol, c; III; genistein).

3 RESULTS

3.1 MINK

The relative food consumption of the mink exposed to β-sitosterol increased in the second month (IV; Table 1). The β-sitosterol-exposed females had larger litters than the control females (P=0.041 T-test; I). The exposed females were heavier than the control females on the first postnatal day, while the BW of the exposed kits was smaller from the first to the twenty-first

postnatal day. The average relative testicular weight of the exposed male kits was higher than that of the control kits. Furthermore, the average relative weight of the prostate glands of the β-sitosterol-exposed kits was higher and the average relative weights of the uteri lower than those of the control kits.

The levels of plasma testosterone in the exposed males increased before mating, which took place on January 13, while those of plasma LDL fell (I). Exposure to phytoestrogens reduced plasma T_4 concentrations in the kits (IV). Genistein exposure reduced the plasma DHT levels in the male kits (I) and increased the plasma ghrelin levels in all kits (IV). Exposure to β-sitosterol reduced the levels of plasma leptin and total protein.

3.2 MICE

Genistein treatment reduced the relative food consumption of the female mice at one and five weeks and that of the male mice at five weeks (III). Exposure to phytosterol occasionally had the same effect, except during the first week when relative food consumption increased (V). The average relative kidney weights of the female pups were lower after exposure to genistein (III). The genistein-exposed male pups had greater average relative weights of the prostate gland and the seminal vesicles than the control pups. Differences in the organ weights were minor and possibly not caused by exposure (II, V). Genistein treatment reduced the plasma estradiol concentrations in the adult males and increased the levels of plasma HDL cholesterol and triglyceride in the adult females (III). The plasma ghrelin concentrations in the adult female mice treated with genistein fell. Genistein increased the plasma triglyceride levels in the male pups and the T_3 levels in the female pups. Exposure to phytosterols increased the plasma leptin concentrations in the males (V).

4 DISCUSSION

4.1 FOOD CONSUMPTION

Recent studies have shown that phytoestrogens can affect the food consumption of experimental animals. For example, phytosterols such as β-sitosterol, and phytosterol esters can increase the food consumption of rats (Hepburn et al., 1999; Kim et al., 2002) and field voles (Nieminen et al., 2003c). This was also seen in mice (V; Ryökkynen, Mustonen, Nieminen unpublished data) and in mink (IV) in the early stages of the experiments. It might be partly explained by the inhibition of cholesterol absorption by phytosterols (Sanders et al., 2000), since the animals would try to compensate for reduced energy (i.e. lipid) intake by increasing their total food intake. However, genistein reduced the relative food consumption of mice in weeks one and five (III) and the same phenomenon has been observed in female rats (1250 ppm, Delclos et al., 2001; 0.1% during gestation, Casanova et al., 1999). Furthermore, the increase in food consumption of genistein-exposed female mice during gestation was less than that of the control females (Wisniewski et al., 2005). On the other hand, in some studies even higher genistein doses had no effect on the food consumption of male rats (25-250 mg/kg BW/day, Wang et al., 2002). There were no differences in the food consumption between control groups and genistein groups of mink (IV). It has been shown that genistein treatment can reduce the weight of adipose tissue and the expression of lipoprotein lipase mRNA in mice (Naaz et al., 2003) and it has also been suggested that many phytoestrogens have significant antiobesity effects (Awad et al., 1999; Bhathena and Velasquez, 2002).

4.2 REPRODUCTION

It is evident that phytoestrogens cause infertility in many animal species, such as the sheep (Bennetts et al., 1946), the California quail (*Lophortyx californicus*, Shaw 1798; Leopold et al., 1976), the

mouse (East, 1955; Jefferson et al., 2005), the cheetah (*Acinonyx jubatus*, Setchell et al., 1987) and the rat (Nagao et al., 2001). Dietary phytoestrogens may have similar effects on the development and fertility of other species, including humans. Human data are very limited and mostly based on clinical studies on the effects of phytoestrogens on plasma lipid and hormone concentrations (for a review see, Moghadasian and Frohlich, 1999; Munro et al., 2003). Most experimental studies have been carried out with laboratory animals and, consequently, the results may not be directly applicable to humans. However, data from experimental animal studies are important when assessing the environmental risks of phytoestrogens.

Table 1. Summary of the effects of genistein, β-sitosterol and phytosterols in mink and mice. ↑ = increase, ↓ = decrease, 0 = no effect, ♀ = female, ♂ = male, nd = not determined, BW = body weight, the four figures or arrows in the columns of the mouse exposed to phytosterols represent successive generations (F_0-F_3 in adults and F_1-F_4 in pups).

	Mink				Mouse			
	Genistein		β-Sitosterol		Genistein		Phytosterols	
	Adults	Kits	Adults	Kits	Adults	Pups	Adults	Pups
Relative food consumption	0		↑		↓		↑/↓	
No. of offspring/litter	0		↑		0		0000	
Postnatal BW at birth		↓		↓		↑		0000
BW day 7		↓		↓		0		↑0↑↓
BW day 14		↓		↓		0		00↑0
BW day 21	0	↓	0	↓	0	0	0000	↑0↑↓
Length	0	↓	0	↓	0	0	0000	↑000♂
Liver/BW	0	0	0	0	0	0	000↑♂	000↓♂
Kidney/BW	0	0	0	0	0	↓♀	0↑↓0♂	0↑0↑♂
Spleen/BW	0	0	0	0	0	0	nd	nd
Thyroid glands/BW	0	0	0	0	nd	nd	nd	nd
Adrenal glands/BW	0	0	0	0	0	0	0000	0000
Testes/BW	0	↑	0	↑	0	0	0000	0↓↑0
Prostate/BW	0	0	0	↑	0	↑	0000	0↑00
Uterus/BW	0	0	0	↓	0	0	0↓00	0↓0↓
Ovaries/BW	0	0	0	0	0	0	0↓00	0↑00
Estradiol	0	0	0	0	↓♂	0	0000	00↑0
Testosterone	↑♂	0	↑♂	0	0	0	0000	0↑0↑
Testicular testosterone ♂	0	0	0	0	0	0	0000	0↑00
Dihydrotestosterone ♂	0	↓	0	0	nd	nd	nd	nd
T_3	0	0	0	0	0	↑♀	0000	0000
T_4	0	↓	0	↓	0	0	0000	0000
Leptin	0	0	0	↓	nd	nd	↑0↑↑	0↑00
Ghrelin	0	↑	0	0	↓♀	0	0000	0000
Total cholesterol	0	0	0	0	0	0	000↓	00↑0
HDL-cholesterol	0	0	0	0	↑♀	0	0000	00↑0
LDL-cholesterol	↓/0*	0	↓/0♂*	0	0	0	0000	0000
Triglycerides	0	0	0	0	↑♀	↑♂	0000	0000
Glucose	0	0	0	0	0	0	0000	↑000
Total protein	0	0	0	↓	nd	nd	nd	nd

* January/June

It is known that genistein is one of the isoflavones that can cause infertility (East, 1955; Setchell et al., 1987; Nagao et al., 2001) and affect adversely the reproductive behaviour of male mice (5 mg/kg/day, Wisniewski et al., 2005) and rats (Flynn et al., 2000). The effect of phytosterols on reproduction is insufficiently known, but no direct adverse effects of dietary phytosterol exposure have been reported in terrestrial mammals. In zebrafish (*Danio rerio*, Hamilton-Buchanan 1822), however, β-sitosterol caused

changes in the sex ratio of offspring and induced vitellogenin production (Nakari and Erkomaa, 2003). Induction of vitellogenin production has also been observed in rainbow trout after a three week exposure to β-sitosterol (25-150 µg/L, Tremblay and Van Der Kraak, 1998). Phytosterols may be partly responsible for reproductive dysfunction in fish, and this has been confirmed by laboratory studies (MacLatchy and van der Kraak, 1995; Lehtinen et al., 1999; Nakari and Erkomaa, 2003).

Subcutaneous β-sitosterol exposure reduced the sperm count in male rats (Malini and Vanithakumari, 1991) and dietary exposure to genistein reduced sperm concentrations and motility in rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792, Bennetau-Pellisero et al., 2001). However, a 7 month dietary exposure to phyto-estrogens did not affect the sperm count of mink (I). This confirms earlier findings in humans exposed to genistein for 2 months (40 mg/day, Mitchell et al., 2001) and in mice exposed to genistein (perinatal 0.1-10 mg/kg/day, Fielden et al., 2003; gavage 2.5 µg/kg BW/day 5 weeks, Jung et al., 2004; gavage 2.5 mg/kg/day or orally 2.5-5 mg/kg/day for 5 weeks, Lee et al., 2004a; Lee et al., 2004b; via drinking water 2.5-25 µg/BW/day, Kyselova et al., 2004). Recent studies have shown that chronic exposure to phytosterols can increase reproductive success in tundra voles (*Microtus oeconomus*, Pallas 1776, Nieminen et al., 2004) and stimulate egg production in hermaphroditic swamp snails (*Lymnaea stagnalis*, Linnaeus), at the expense, however, of egg quality (β-sitosterol 1-100 ng/L, Czech et al., 2001).

In the current study, the β-sitosterol-exposed mink had slightly larger litters (7.0 kits/litter) than the control females (4.4 kits/litter) and the genistein-exposed mink (5.7 kits/litter) (I). The β-sitosterol-exposed mink also had larger litters than those usually found in commercially farmed mink in Scandinavia (5.0 kits/litter, Clausen et al., 1992) and even in mink selected for improved litter size (5.3 kits/litter, Lagerkvist et al., 1993). This finding may be of economic importance in domestic animal production, but could be harmful in nature, where there is competition for limited food resources. It must be remembered that the size of the experimental groups was relatively small (n = 10 pairs/groups and 7-8 females/group gave birth). These findings should, therefore, be regarded as preliminary and more research will be needed to confirm them.

4.3 POSTNATAL DEVELOPMENT AND ORGANS

The mink kits exposed to both phytoestrogens had lower average BW from birth until 21 days of age than the control kits (I). A similar effect has been observed in the postnatal development of mice exposed to genistein (5 mg/kg/day, Wisniewski et al., 2005) and rats exposed to phytostanol (8.76% of diet, Whittaker et al., 1999). In the case of mink, this effect may be partly due to the fact that the exposed females had slightly larger litters than the controls (I). This may have caused their kits to gain less BW because the dams had to feed a larger number of offspring. However, in other phytoestrogen studies exposed offspring were lighter than their controls, even though the experimental groups had the same litter sizes (Whittaker et al., 1999; Wisniewski et al., 2005). A similar effect could be seen also in the BW changes of the female mink (I). After giving birth the exposed females had greater BWs than the controls, but this difference disappeared during lactation. The organ weights of the male mice exposed to the phytosterol mixture showed only minor changes (II), but these were not cumulative over generations.

4.3.1 Reproductive organs

Many mammalian developmental stages are sensitive to disruption by exogenous estrogens and androgens. However, the rate of development of the reproductive system varies among species and it is mainly the

stage at which exposure occurs that determines the subsequent effects. The exact effect of phytoestrogens on the development of the male reproductive system is unclear, but estrogens and estrogen-like substances tend to have demasculinising or anti-androgenic effects (Wisniewski et al., 2003; Svechnikov et al., 2005). At early life stages, this is probably caused by the suppression of testosterone production (Williams et al., 2001) or the loss of androgen receptors (McKinnell et al., 2001). It is known that other hormones, such as thyroid and growth hormone, can affect Sertoli cell proliferation (Sharpe et al., 2000) and, consequently, spermatogenesis. Thus exposure to any factor that changes the production of these hormones may affect the reproductive system through their effects on Sertoli cells. In females, exposure to exogenous estrogens such as phytoestrogens can reduce the production of endogenous estrogens (Setchell et al., 1984). Exposure during early life stages may also have permanent effects. If phytoestrogen exposure occurs during adulthood, it does not necessarily have any significant biological consequences.

Many studies have shown that dietary phytoestrogen exposure can particularly affect the weight of the reproductive organs in experimental animals (I, III; Strauss et al., 1998; Nagao et al., 2001; Wisniewski et al., 2003). For example, uterine weight increased when subcutaneous β-sitosterol was administered to sheep (20 days at 20 mg/animal/day, El Samannoudy et al., 1998) and rats (0.5-5 mg/kg/day, Malini and Vanithakumari, 1993) and a similar effect was observed when subcutaneous genistein was given to rats (16.6-50 µg/g BW, Cotroneo and Lamartiniere, 2001; 2 mg/kg days 1-6, Lewis et al., 2003) and mice (0.7-5 mg/day, Ishimi et al., 2000). Dietary exposure to genistein increased the weight of the uterus in beagle dogs (500 mg/kg/day for 4 and 13 weeks, McClain et al., 2005), cheetahs (genistein/daidzein 50/mg/day, Setchell et al., 1987), rats (150-750 µg/g/day, Santell et al., 1997; 250-1000 mg/kg/day, Cotroneo and Lamartiniere, 2001; 50-100 mg/kg/day, Diel et al., 2004) and mice (2.5 mg/kg/day, Cheng et al., 1954; 300-1500 ppm, Naaz et al., 2003). Genistein administered by gavage increased the weight of the uterus in rats (100-200 mg/kg/day, Stroheker et al., 2003). Mink kits exposed to dietary β-sitosterol showed the opposite results, however, since the weight of the uterus decreased (I). Moreover, mouse pups exposed to phytosterol mixture showed similar results in two of the generations and adult mice in one of the generations (II). These could be taken as indications of endocrine disruption of the reproductive organs, observable even at the macroscopic level. The differences between studies could be partly explained by the different routes of exposure. However, the exposure of mink and mice to genistein did not affect the weight of the uterus (I, III), although it has been observed in other species that the uterine weight increased after dietary exposure to genistein at larger doses (25-750 mg/kg/day; Setchell et al., 1987; Santell et al., 1997; Cotroneo et al., 2001; Diel et al., 2004; McClain et al., 2005).

Perinatal exposure to genistein increased the weight of the testes in rats in adulthood (orally 25-100 mg/kg/day, Nagao et al., 2001) and a similar increase was observed in the testicular weight of mink kits exposed to phytoestrogen (I). It has been observed that the testicular weight decreased in rats given subcutaneous β-sitosterol (Malini and Vanithakumari, 1991) or neonatally injected subcutaneous genistein (Atanassova et al., 2000), and in 30-day-old mice exposed to genistein via drinking water (Kyselova et al., 2004). Testicular size (length and width) decreased in mice given dietary genistein treatment (Wisniewski et al., 2003).

The weight of the prostate gland increased in mink kits exposed to β-sitosterol (I) and in mouse pups exposed to genistein (III). Exposure to genistein via drinking water (25 µl/kg body weight/day) has been shown to reduce the weight of the

prostate and the seminal vesicles in adult and 30-day-old mice (Kyselova et al., 2004). The same effect has been observed in the weight of the ventral prostate in rats after dietary genistein exposure (1250 ppm, Delclos et al., 2001) and subcutaneous β-sitosterol exposure (Malini and Vanithakumari, 1991), while the opposite effect was found in rats exposed neonatally to genistein (12.5-100 mg/kg/day, Nagao et al., 2001) or exposed chronically to genistein until they were 70 days old (5-300 mg/kg/day, Wisniewski et al., 2003). However, it seems that these effects of phytoestrogens on the weights of the ovaries and the uterus may disappear in adulthood (Awoniyi et al., 1998). Further studies are needed to determine whether the changes in the reproductive organs of the experimental animals are lasting or transient, as observed earlier in rats (Awoniyi et al., 1998). If enduring, such effects could be detrimental to the reproductive health of the individual.

4.4 ENDOCRINE VARIABLES

4.4.1 Phytoestrogens and estrogen receptors

It has been shown that the phytoestrogens used in this study can bind to estrogen receptors (ER, Mellanen et al., 1996; Kuiper et al., 1998; Casanova et al., 1999; Tollefsen et al., 2002). However, the relative binding affinity is much higher for genistein than for β-sitosterol (Kuiper et al., 1997; Kuiper et al., 1998). Both ERα and ERβ have been identified in mammals. These subtypes are distributed in many tissues, including the reproductive system, i.e. the mammary gland, the uterus, the ovaries, the testes and the prostate. However, the tissue distribution and relative binding affinities of ERα and ERβ differ. For example, ERβ can be found in the brain, the thymus, the bone, the bladder, the prostate, and the vascular epithelia (Kuiper et al., 1997). It has been shown that the relative binding affinity of genistein is much higher for ERβ than ERα and that it is the inverse of the relative binding affinity of 17α-estradiol, the endogenous mammalian hormone (Kuiper et al., 1997; Kuiper et al., 1998; Kostelac et al., 2003). Thus, genistein signalling through ERβ may be important for its biological actions. However, the relative binding affinity of β-sitosterol for ERα and ERβ is extremely weak (Kuiper et al., 1997). It has been observed that β-sitosterol has estrogenic activity in T-47D the breast tumour cell line but not in MCF-7 cells (Mellanen et al., 1996) and that these cell lines do not have ERβ (Kuiper et al., 1997). Genistein also inhibits the enzymes required for androgen metabolism (Evans et al., 1995; Weber et al., 1999; Fritz et al., 2003) and androgen receptor (AR) gene expression in rodents (Fritz et al., 2002b),whereas reduced AR expression is mediated through ER-β (LNCaP; Bektic et al., 2004). Moreover, the levels of dorsolateral prostate ERβ decreased in rats exposed to dietary genistein (5-500 ppm, Dalu et al., 2002). However, recent studies have shown that the effects of genistein on the thymus, for example, are only partially mediated through ER (Yellayi et al., 2002; Yellayi et al., 2003). Thus, it is surmised that phytoestrogens may cause their effects directly via ERs or indirectly via still unknown mechanisms.

4.4.2 Sex hormones

Steroid hormones are derived from cholesterol. Phytosterols are structurally similar to cholesterol and thus they may act as precursors of sex steroids (Werbin et al., 1960; Svoboda et al., 1967). Testosterone is synthesized in the testes and, in small quantities, in the ovaries (Hadley, 2000). It is an intermediate in estradiol synthesis and can be converted to dihydrotestosterone (DHT) by the cytoplasmic enzyme 5α-reductase. Testosterone and other androgens promote protein synthesis and the growth of tissues with ARs. Estradiol is an endogenous hormone that is mainly produced in the ovaries and the testes. It affects the growth, differentiation and

functioning of many target tissues such as reproductive organs.

Phytoestrogens have potential effects on the endocrine system of animals, through the ER and/or AR (Fritz et al., 2002b; Cotroneo et al., 2001). As observed previously, dietary phytoestrogen exposure affects the plasma sex hormone levels of experimental animals. For example, the levels of plasma estradiol and testosterone increased in male field voles exposed to dietary phytosterol mixture (5 mg/kg BW/day, Nieminen et al., 2003c). On the other hand, plasma and testicular testosterone levels decreased in adult male mice exposed to subcutaneous genistein (Strauss et al., 1998) and in adult male tundra voles exposed to dietary phytosterol (5 mg/kg BW/day), whereas the levels of testicular testosterone in their 21-day-old offspring had increased (Nieminen et al., 2004). Phytosterol exposure also reduced the concentrations of plasma testosterone in brook trout (*Salvelinus fontinalis*, Mitchill 1814, 72% β-sitosterol via intraperitoneal implants 20-100μg/g, Gilman et al., 2003), goldfish (*Carassius auratus auratus*, Linnaeus 1758, β-sitosterol injection 20-100 μg/g; MacLatchy and van der Kraak, 1995) and rats (dietary phytosterol mixture, Awad et al., 1998). Perinatal exposure to genistein (5-300 mg/kg/day) reduced the concentrations of plasma testosterone in male rats in adulthood (Wisniewski et al., 2003), whereas life-long genistein treatment increased them (25-250 mg/kg diet, Fritz et al., 2002b; 500 mg/kg/day, Dalu et al., 2002).

In this study the levels of plasma testosterone in adult male mink exposed to phytoestrogen increased at an earlier date (I) than would occur naturally at the onset of the mating season (Sundqvist et al., 1988). It has been shown that exposure to genistein accelerated testicular development in male, but delayed gonadal development in female rainbow trout (500-1000 ppm, Bennetau-Pellisero et al., 2001) and in female Japanese medaka (1000 μg/L, *Oryzias latipes*, Temminck and Schlegel 1846, Kiparissis et al., 2003). A similar accelerated maturation of males and slower maturation of females has been observed in brown trout (*Salmo trutta lacustris*, Linnaeus 1758) after a 4.5-month exposure to phytosterols, mainly β-sitosterol (10 and 20 μg/L, Lehtinen et al., 1999).

In mink kits, perinatal genistein exposure reduced the levels of plasma DHT (I) required for the sexual differentiation of males. This may be mainly caused by reduced activity of 5-α-reductase, as observed in human genital skin fibroblast monolayers and benign prostatic hyperplasia tissue homogenates (Evans et al., 1995), or it may be partly caused by increased 3-α/β-hydroxysteroid dehydrogenase activities, which convert DHT to 5-α-androstane[-3-α or 3-β],17-β-diol (Wilson et al., 2002). On the other hand, dietary exposure to an isoflavone mixture (mainly genistein 60 %, 300 mg/kg/day) did not cause significant changes in the plasma DHT or testosterone levels of adult rats (Kwon et al., 2001). Plasma DHT levels have even been shown to increase in human males, after two or four weeks of Trinovin treatment (40 mg/day, includes isoflavones such as genistein, daidzein, biochanin and formononetin; Lewis et al., 2002), and in male rats at the age of 140 PND after dietary genistein exposure (100-500 mg/kg/day, Dalu et al., 2002). Dietary phytosterol exposure has also been shown to reduce 5-α-reductase activity in the livers and prostate glands of rats (Awad et al., 1998).

A reduction in plasma DHT levels may be associated with the promotion and progression of prostate diseases (Tham et al., 1998). On the other hand, many studies have demonstrated that phytoestrogens can alleviate the symptoms of prostatic diseases (Berges et al., 1995; Schleicher et al., 1999; Wang et al., 2002; Dalais et al., 2004). It has been shown that DHT increased the relative weight of the dorsolateral prostate in rats (Fritz et al., 2002a). Thus, lowered androgen levels may offer protection against prostate cancer. However, in these

studies the prostate weight of mink kits was unaffected by genistein (I), whereas in mouse pups the weights of the prostate glands and the seminal vesicles increased (III). This is confirmed by other rodent studies (Delclos et al., 2001; Kyselova et al., 2004). It seems that perinatal exposure to phytoestrogens, as observed in this study (I), may have a greater effect than exposure during adulthood. Exposure to phytoestrogens reduced plasma estradiol and progesterone levels in 21-day-old rats but the effects disappeared in adulthood (70 days old, Awoniyi et al., 1998). Further research is needed to determine whether the observed effects are constant in mink. However, the observed decrease in the plasma DHT concentrations of the mink kits exposed to genistein may be an indication of endocrine disruption.

4.4.3 Thyroid hormones

Thyroid hormones affect growth, development, metabolism, body temperature regulation and circulation (Hadley, 2000). Their tissue effects are mediated mainly via T_3, while T_4 acts as its precursor. Both hormones are synthesised in the thyroid gland and about 80% of its production consists of T_4. T_3 is also produced by the peripheral monodeiodination of T_4. Of the two hormones, T_3 has greater biological activity. It has been shown that natural estradiol operates directly on thyroid tissue through ER (Chen and Walfish, 1978). The phytoestrogens used in this study can also bind to ER (Casanova et al., 1999; Kuiper et al., 1997; Mellanen et al., 1996), possibly affecting the secretion of thyroid hormones. It has been shown that genistein possesses goitrogenic activity and causes reduced thyroid peroxidase activity *in vivo* (0.4-40 mg/kg/day, Chang and Doerge, 2000; Doerge and Sheehan, 2002) and *in vitro* (Divi et al., 1997). Thyroid peroxidase is essential to normal thyroid function as it catalyses the reactions required for thyroid hormone synthesis. Dietary consumption of genistein also increases intrathyroidal genistein concentrations in rats in a dose-dependent manner (Chang and Doerge, 2000) and may therefore lead to reduced thyroid function.

It has been observed that estradiol treatment causes reduced T_4 secretion in rats (Chen and Walfish, 1978), just as phytoestrogens do in mink kits (IV) and golden Syrian hamsters (28-days exposure to soy protein, *Mesocricetus auratus*, Waterhouse 1839; Wright and Salter, 1998). These findings conflict with earlier observations of increased plasma thyroid hormone levels after phytoestrogen exposure (for review see, Forsythe, 1995). Moreover, exposure to genistein increased plasma T_3 levels in 21-day-old female mice (III). The plasma T_4 concentrations were also slightly higher in the genistein-exposed female mice pups than in the control pups (III). Thus, the change in plasma T_3 levels was probably not caused by the peripheral monodeiodination of T_4, but possibly indicates increased thyroid hormone synthesis in the thyroid gland due to the genistein exposure. A similar increase in the levels of plasma T_3 has been found earlier in male golden Syrian hamsters after a 28-day exposure to a soy protein diet (Wright and Salter, 1998), in 65-day-old and 110-day-old Long-Evans male rats after an isoflavone-rich diet (Lephart et al., 2004a) and in female raccoon dogs (*Nyctereutes procyonoides*, Gray 1834) after 2- and 4- week dietary exposures to phytosterols (8 mg/kg/day, Nieminen et al., 2003a).

The observed changes in plasma T_3 and T_4 levels after phytoestrogen exposure may cause undesirable effects later in life, unless they are transitory (III, IV), as has been observed earlier with respect to the levels of sex hormones in rats exposed to genistein (Awoniyi et al., 1998). These effects could impact on growth, development, metabolism, temperature regulation and circulation (Hadley, 2000). However, some of the changes in the levels of thyroid hormones may be partly explained by other factors. For example, the smaller BW of

phytoestrogen-exposed mink kits may be partly caused by the reduced plasma T_4 concentrations, possibly acting together with the larger litter size (I, IV).

4.4.4 Weight regulation hormones

Leptin is a ~16 kDa protein hormone secreted mostly by the white adipose tissue (Zhang et al., 1994; Friedman and Halaas, 1998). Ghrelin is a 28-amino acid peptide hormone expressed mainly in the stomach and, at lower concentrations, found in the small and large intestines, the hypothalamus, the pituitary, the kidneys, the placenta and the α-cells of the pancreatic islets (Kojima et al., 1999; Date et al., 2000; Tschöp et al., 2000; Horvath et al., 2001). Both hormones regulate food intake. Leptin inhibits appetite (Friedman and Halaas, 1998) and ghrelin stimulates it (Tschöp et al., 2000). It has been suggested that leptin may act as a signal substance between adipose tissue and the reproductive system (Mounzih et al., 1997; Spicer, 2001; Moschos et al., 2002; Sagawa et al., 2002).

It has been shown recently that a phytoestrogen rich-diet reduces the levels of plasma leptin in male rats (Lephart et al., 2004a; Lephart et al., 2004b). Genistein can also reduce leptin secretion from rat adipocytes and this can been seen as a reduction in the plasma leptin levels (Szkudelski et al., 2005). A similar result was observed in mink kits exposed to β-sitosterol (IV), but the opposite was found in adult male mouse plasma after exposure to phytosterols (V). The positive correlation between BW and plasma leptin levels seen in adult male mice fits the established pattern of a direct relationship between plasma leptin levels and body adiposity, with leptin acting as a satiety signal (Halaas et al., 1995; Maffei et al., 1995; Friedman and Halaas, 1998). In addition, the food intake of PS-males correlated negatively with the plasma leptin levels when all the studied generations were analysed together (V). It has been observed that plasma leptin levels in human males decrease after puberty, since elevated testosterone levels inhibit the secretion of leptin (Hislop et al., 1999). Thus, PS may prevent the inhibition of leptin secretion by testosterone, but this was not apparent in the present study, since there were no differences in the plasma or testicular testosterone levels between groups (II).

The plasma ghrelin concentrations decreased in adult female mice (III) but increased in mink kits exposed to genistein (IV). A reduction in plasma ghrelin levels has also been observed in adult European polecats after sub-acute phytosterol exposure (50 mg/kg/day, Nieminen et al., 2003b). Reduced levels of plasma ghrelin inhibit appetite (Tschöp et al., 2000) and this was observed in adult female mice exposed to genistein (III). It has been shown that ghrelin may be a factor in the control of testicular function (Tena-Sempere et al., 2002). Both ghrelin and leptin have been shown to have an inhibitory effect on testicular testosterone secretion (Tena-Sempere et al., 1999; Tena-Sempere et al., 2002). However, such interactions between these hormones were not seen in this study (I-V).

4.5 BIOCHEMISTRY OF PLASMA

It is evident based on clinical experiments that phytoestrogens may reduce the risk of cardiovascular disease through an improved blood lipid profile (for review see, Demonty et al., 2003; Kritchevsky and Chen, 2005; Thompson and Grundy, 2005). Phytoestrogens are marketed, therefore, as being able to lower the elevated serum total cholesterol and LDL cholesterol levels. It has also been shown in clinical studies that genistein and other isoflavones can increase serum HDL cholesterol levels in females (Demonty et al., 2003), as observed in adult female mice exposed to genistein (III). In addition, isoflavone exposure can reduce the serum triglyceride levels in experimental animals (Forsythe, 1986). On the other hand, clinical studies have demonstrated that phytosterols sometimes have no effect on serum HDL cholesterol or triglyceride levels (Demonty et al., 2003).

Phytoestrogen exposure had minimal effects on the plasma lipid profile of mink and mice (III, IV, V). The minor changes observed could be partly explained by the small doses compared to those used in previous studies (Peterson, 1958).

Due to some unknown mechanism, plasma total protein concentrations decreased in β-sitosterol-exposed mink kits, as has been similarly observed in rats after subcutaneous β-sitosterol exposure (Malini and Vanithakumari, 1990). It should be emphasized that results obtained from mice or mink cannot be directly extrapolated to human risk assessment, since we have a different plasma lipid profile (Alexander and Day, 1973; Chapman, 1980; Nikitin et al., 1982). HDL cholesterol is a major lipoprotein class in mice (70-86%, Camus et al., 1983) and mink (~ 80%, Nikitin et al., 1982), but not in humans (17%, Henry, 1974). In rats, HDL cholesterol is also the most important vehicle for the transport of dietary sterols, especially plant sterols (80% of sitosterol were carried in HDL, Sugano et al., 1978). Phytoestrogens lower cholesterol in human serum, and this is their most important application, but they have only minor effects on the plasma lipid profile of mink and mice, even after long-term exposure (III-V).

4.6 METABOLIC ENZYMES

Exposure to phytoestrogens also affected the carbohydrate metabolism of mink (IV). For example, in the exposed groups glycogen concentrations increased in adult mink kidneys. Recently, it has been reported in European polecats that subchronic phytosterol exposure (50 mg/kg/day) increased liver glycogen concentrations in a similar manner (Nieminen et al., 2003b) while subcutaneous β-sitosterol injections also increased glycogen concentrations in the uteri of rats (Malini and Vanithakumari, 1992). Furthermore, the activity of the glycogen phosphorylase increased in the muscles of phytoestrogen-exposed adult mink and in the livers of genistein-exposed mink kits, indicating increased glycogenolysis. Similar changes in the kidney phosphorylase activity of female European polecats (Nieminen et al., 2003b) and field voles (Nieminen et al., 2003c) have been observed after subchronic phytosterol exposure.

5 CONCLUSION

The results of this study partly confirm earlier observations that dietary exposure to phytoestrogens can cause significant effects on experimental animals. It seems that phytoestrogen exposure to early life stages produced the most marked effects. Furthermore, exposure to phytoestrogens caused stronger responses in mink, which are carnivorous, than in mice, which are omnivorous and have, during evolution, become adapted to phytoestrogens via their diet. The most significant findings concerning exposure to phytoestrogens were observed in mink kits, including reduced concentrations of plasma T_4 (IV) and changes in the weights of reproductive organs (I). In adult individuals, the onset of the testosterone peak was accelerated in phytoestrogen-exposed male mink before the mating season (I) and the plasma leptin levels increased in male mice after phytosterol exposure (V). Exposure to phytoestrogens had no harmful effects on the reproduction of the experimental animals. Future research needs to clarify the specific mechanisms behind these findings.

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