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**EFFECTS OF BISPHENOL A AND
PHYTOSTEROLS ON THE EUROPEAN
POLECAT (*Mustela putorius*) AND THE FIELD
VOLE (*Microtus agrestis*)**

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ACADEMIC DISSERTATION

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ABSTRACT

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Endocrine disruptors are exogenous substances with adverse health effects due to changes in endocrine functions. Environmental estrogens have the capability to bind to the estrogen receptor. They are hypothesized to be a cause of falling sperm counts in men and breast cancer in women. In nature, endocrine disruption causes intersexuality and reproductive disturbances in fish. Bisphenol A (BPA) is a synthetic compound used in the production of plastics. It has estrogenic activity *in vitro*. Phytosterols and –stanols (PS) are the analogues of animal cholesterol in plants. PS are ingested by the western population in cholesterol-lowering spreads and health products and enter the ecosystem via pulp mill effluents. Also PS have potential estrogenic effects.

The aim of the study was to expose a carnivore (polecat) and a herbivore (field vole) to BPA and PS in a subacute exposure and to determine the effects of these compounds on endocrine parameters and selected enzyme activities of biotransformation and carbohydrate and lipid metabolism. The studies aimed to determine whether BPA or PS were estrogenic *in vivo*, what their other hormonal and metabolic effects were and if suitable biomarkers for environmental monitoring could be found. The studies also aimed to provide preliminary data for chronic exposure studies. The polecats were exposed to BPA and PS perorally for two weeks. The exposure of the field voles to PS was similar to this, but BPA was administered to the voles subcutaneously for four days.

BPA increased the plasma testosterone concentrations slightly in polecats and significantly in field voles. The activities of liver UDP-glucuronosyltransferase and glutathione S-transferase (GST) increased in female polecats, but the liver ethoxyresorufin *O*-deethylase and GST activities decreased in field voles. The mortality of field voles increased significantly due to BPA exposure.

PS caused an increase in the plasma estradiol and testosterone concentrations of polecats. The plasma ghrelin levels decreased. The liver glycogen content and glucose-6-phosphatase activity increased, but the liver lipase esterase activity decreased. The serum low-density lipoprotein cholesterol levels increased in polecats. In field voles the effects of PS were mostly biphasic with a change in hormone concentration or enzyme activity at a lower PS dose with a return to the levels of the control animals at a higher PS dose. PS caused no clear effect on biotransformation enzymes, but an increase in food intake was observed in field voles.

All the effects of BPA or PS do not seem to be due to estrogenicity. A common effect was an increase in the circulating testosterone concentrations. PS caused also an increase in estradiol levels. The effects of PS on the endocrine system were more pronounced than the effects of BPA and they could be due to increased sex steroid synthesis from PS precursors. The effects of BPA were more pronounced on the biotransformation enzymes. PS do not seem to be recognized as foreign compounds and they can affect the organism without interference from the biotransformation apparatus. No reliable biomarker could be found as the effects were widespread but unspecific. Yet BPA affected the polecats below the oral reference dose of 50 mg kg⁻¹ d⁻¹ considered to be without deleterious effects. PS also caused previously unreported effects at doses used to lower elevated serum cholesterol levels in humans.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to by their Roman numerals:

- I Nieminen P, Lindström-Seppä P, Juntunen M, Asikainen J, Mustonen A-M, Karonen S-L, Mussalo-Rauhamaa H, Kukkonen JVK 2002: *In vivo* effects of Bisphenol A on the polecat (*Mustela putorius*). *J Tox Environ Health*, in press.
- II Nieminen P, Lindström-Seppä P, Mustonen A-M, Mussalo-Rauhamaa H, Kukkonen JVK 2002: Bisphenol A affects endocrine physiology and biotransformation enzyme activities of the field vole (*Microtus agrestis*). *Gen Comp Endocrinol*, in press.
- III Nieminen P, Mustonen A-M, Lindström-Seppä P, Asikainen J, Mussalo-Rauhamaa H, Kukkonen JVK 2002: Phytosterols act as endocrine and metabolic disruptors in the European polecat (*Mustela putorius*). *Tox Appl Pharmacol* 178: 22-28.
- IV Nieminen P, Mustonen A-M, Lindström-Seppä P, Kärkkäinen V, Mussalo-Rauhamaa H, Kukkonen JVK 2002: Phytosterols affect endocrinology and metabolism of the field vole (*Microtus agrestis*). Manuscript submitted to *Exp Bio Med*.

ABBREVIATIONS

AMP	Adenosinemonophosphate
ANOVA	Analysis of variance
BM	Body mass
BMI	Body mass index
BPA	Bisphenol A
DDT	Diphenyltrichloroethane
DNA	Deoxyribonucleic acid
EC ₅₀	Median effective concentration
EDTA	Ethylenediaminetetraacetic acid
ELISA	Immonoassay
EPA	Environmental Protection Agency
EROD	Ethoxyresorufin <i>O</i> -deethylase
FSH	Follicle stimulating hormone
G6Pase	Glucose-6-phosphatase
GST	Glutathione S-transferase
HDL	High density lipoprotein
ip	Intraperitoneal
IRMA	Immunoradiometry
LC ₅₀	Median lethal concentration
LDL	Low density lipoprotein
LH	Luteinizing hormone
NPY	Neuropeptide Y
PCB	Polychlorinated biphenyl
PS	Phytosterols or plant sterols
RIA	Radioimmunoassay
sc	Subcutaneous
SD	Standard deviation
SE	Standard error
T ₃	Triiodothyronine
T ₄	Tetraiodothyronine
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TSH	Thyroid stimulating hormone
UDPGT	UDP-glucuronosyltransferase

1 INTRODUCTION

"An endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function" (Commission of the European communities, 1999). Principal targets of these xenobiotics are presumably steroid receptors, especially estrogen and androgen receptors (Luster et al., 1984). Steroids (and their disruptors) bind to their cognate receptors inducing a conformational change in the receptor leading to modulation of transcription (Perlmann and Evans, 1997). Environmental estrogens are chemically synthesized compounds, e.g. organochlorides (polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT)), etc. The effects of environmental estrogens or xenoestrogens are determined by the receptors with which they interact, by the tissue distribution of these receptors, and the context of the estrogen responsive elements on the DNA in the target cell (Limbird and Taylor, 1998). In addition, many xenobiotics with potential for endocrine disruption have also other deleterious effects that can be directly toxic and targeted at many organ systems (Atkinson and Roy, 1995a,b) or at biotransformation (Atkinson and Roy, 1995a; Hanioka et al., 1998, 2000).

At present, there are more than 87 000 chemicals with endocrine disrupting potential in use (US EPA, 1998). This presents the scientific community with a formidable screening process; yet there are plans to screen at least 15 000 of these agents (Macilwain, 1998). Humans can ingest natural or chemical estrogens from various sources (Sharpe and Skakkebaek, 1993). Estrogens can be endogenous or synthetic (e.g. oral contraceptives). There are also many food plants with weak estrogens causing an increase in the production of sex hormone binding globulin (SHBG) leading to reduced exposure to endogenous estrogens (Adlercreutz et al., 1987).

Environmental estrogens have been hypothesized to be involved in the falling sperm counts and disorders of the male reproduction that have increased in incidence over the last 40-50 years (Giwercman and Skakkebaek,

1992; Sharpe and Skakkebaek, 1993). Organochlorine residues have been linked to the risk of breast cancer in women (Wolff et al. 1993). There is worldwide evidence for endocrine disruption in nature due to the exposure of aquatic animal populations to various chemicals. In Sweden a 80% reduction in gonadosomatic index has been observed in perch (*Perca fluviatilis*) in a lake near a public refuse dump (Noaksson et al., 2001). Also the sexual maturity of female perch has arrested to a nonreproducible immature stage. In the United Kingdom there is a high incidence of intersexuality in wild roach (*Rutilus rutilus*) supposed to be due to estrogenic contaminants (Jobling et al., 1998). Hepatic biotransformation of testosterone of juvenile alligators (*Alligator mississippiensis*) in a contaminated lake in Florida, USA, has been disrupted (Gundersen et al., 2001). Similar effects in fish have been observed also e.g. in Canada (Munkittrick et al., 1992) and Central Europe (Van der Oost et al., 1994).

2 REVIEW OF THE LITERATURE

2.1 Bisphenol A

Bisphenol A (BPA) or 2,2-bis(4-hydroxyphenyl)propane (Fig. 1) is a compound used widely in the production of polycarbonate and other plastics and flame retardants with an annual production exceeding 420 000 tons (Alexander et al., 1988). Final products include adhesives, coatings, paints, building materials, thermal paper, etc. (Staples et al., 1998). BPA is a solid substance under ambient conditions, and it can be purchased as crystals, prills or flakes. BPA waste may enter the environment during handling, loading and unloading, heating, as accidental spills or releases. BPA also leaches out in trace amounts from resins and polycarbonate plastics of food packages (Knaak and Sullivan, 1966; Krishnan et al., 1993).

The solubility of BPA in water is 120-300 mg l⁻¹ (Bayer Leverkusen, 1989). BPA is considered to have only low potential for bioaccumulation or biomagnification (Gillette,

1983; US EPA, 1995). BPA is degraded quite easily in biological waste treatment systems (Staples et al., 1998). For instance, the activated sludge treatment removes >99% of BPA in two weeks (Furun et al., 1990). In waste treatment systems this seems to be achieved by a gram-negative aerobic *bacillus* of the MV1 strain (Lobos et al., 1992). As a result, 60% of the carbon in BPA is mineralized to CO₂, 20% associates with the bacterial cells and 20% is converted to soluble organic compounds. There is also a possibility for photodegradation of BPA in the atmosphere as well as in surface waters with a half-life of 0.7-7.4 hours in air and 2.5-66 days in water (Staples et al., 1998).

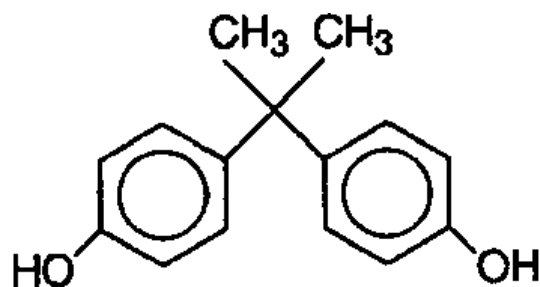


Fig. 1. The chemical structure of bisphenol A.

BPA concentrations in natural waters in Europe are mostly undetectable (Hendriks et al., 1994). In Japan, BPA concentrations in various industrial and pristine waters are usually between 0.06-0.11 g l⁻¹ (Matsumoto, 1982), but as high as 0.17 g l⁻¹ in waste landfill leachates (Yamamoto et al., 2001).

The toxicity of BPA to aquatic organisms is slight to moderate. The median effective concentration (EC₅₀) of *Daphnia magna* is approximately 10 mg l⁻¹ (Alexander et al., 1988). The median lethal concentration (LC₅₀) for freshwater fish is about 4.7 mg l⁻¹ for the minnow (*Pimephales promelas*) (Alexander et al., 1988) and about 4.0 mg l⁻¹ for the rainbow trout (*Oncorhynchus mykiss*) (Staples et al., 1998). In zoospores of the fungus *Aphanomyces cochlioides* BPA causes repellent activity (Islam and Tahara, 2001). In eukaryotes – algae and freshwater invertebrates – the concentrations producing chronic effects are approximately the same as the

concentrations causing acute effects. Prokaryotes are less affected. For instance, the growth of *Pseudomonas putida* is attenuated by only 10% at a BPA dose exceeding >320 mg l⁻¹ (Staples et al., 1998).

BPA conjugates with glucuronic acid in liver microsomes (Yokota et al., 1999; Snyder et al., 2000). BPA glucuronide is also the major metabolite in urine (Knaak and Sullivan, 1966). The cytochrome P450 system is closely associated with the metabolism and clearance of BPA (Atkinson and Roy, 1995a). BPA at 20-40 mg kg⁻¹ d⁻¹ intraperitoneally (ip) suppresses the activity of male specific P450 isoforms *in vivo* in rats (Hanioka et al., 1998, 2000). In humans hepatic CYP2C8 and 2C19 are also inhibited (Niwa et al., 2000). BPA is excreted into milk in rats (Yoo et al., 2001) as BPA glucuronate (Snyder et al., 2000).

Exposure to high doses of BPA causes toxicity in multiple organ systems such as the kidney, liver, spleen and pancreas (Atkinson and Roy, 1995a,b). BPA is concentrated in lungs (Yoo et al., 2000) and brown adipose tissue (Nunez et al., 2001). It decreases rat body mass (BM or body weight) gain without any effect on food intake indicating effects of BPA on energy balance. BPA exposure is also associated with an increase in the amount of cancers in the hematopoietic system in rodents (Ashby and Tennant, 1988; Roy et al., 1997). High BPA doses also cause reproductive toxicity and affect cellular development in rats and mice (Morrissey et al., 1987). In rats ip BPA decreases BM (Hanioka et al., 1998). The targets of the toxic effects of BPA are probably mitochondria and mitochondrial respiration (Nakagawa and Tayama, 2000).

BPA binds to estrogen receptors and activates them (Krishnan et al., 1993). Together with many alkylphenols BPA has estrogenic activity in human cultured MCF-7 breast cancer cells. In conventional *in vitro* media the relative binding affinity of BPA to the estrogen receptor is very low (0.006%), but the affinity increases significantly in serum (Nagel et al., 1997). High concentrations of BPA also induce growth and prolactin secretion of estrogen-responsive pituitary tumor cell lines (Chun and Gorski, 2000). This requires, however, 1000 nM BPA

to attain the same effects as 0.01 nM estradiol.

All the effects of BPA do not, however, seem to be related to estrogen. BPA, unlike estradiol, inhibits the human chorionic gonadotropin (hCG) stimulated cAMP and progesterone formation in mouse Leydig tumor cells (Nikula et al., 1999). In rats subcutaneous (sc) 14-day BPA exposure at 1 mg animal⁻¹ d⁻¹ decreases the testicular response to hCG resulting in decreased levels of plasma testosterone but increased levels of luteinizing hormone (LH) (Tohei et al., 2001). Peroral BPA in drinking water at 0-10 ppm, however, has no effects on the development of rat reproductive organs (Cagen et al. 1999). Fischer 344 rats are, however, more susceptible to BPA, with increased prolactin release in ovariectomized rats after sc BPA exposure of 40-45 µg kg⁻¹ d⁻¹ and an increase in uterine wet weight at 0.3 mg kg⁻¹ d⁻¹ (Steinmetz et al., 1997, 1998). In mice peroral BPA at 2-20 µg kg⁻¹ d⁻¹ fed to pregnant females increases the adult prostate weight of their offspring (Nagel et al., 1997). BPA also accelerates puberty in female rats (Ashby and Tinwell, 1998).

As BPA is used in many consumer products, it can enter the human body from e.g. reusable baby bottles (Biles et al., 1997), food packaging materials (Krishnan et al., 1993), liquid of canned vegetables (Brotons et al., 1995) and dental sealants (Olea et al., 1996). The entry route of BPA into the organism is, however, of importance. The absolute oral bioavailability of BPA in rats in low (about 5 %) (Yoo et al., 2001) compared to sc administration (Laws and Carey, 1997).

The value of 50 mg kg⁻¹ d⁻¹ is recommended as the oral reference dose of BPA for use in risk assessment of human exposure (U.S. EPA, 1987). It is an estimate of a daily exposure to the human population that is likely to be without an appreciable risk or deleterious effects during a lifetime.

2.2 Phytosterols

Plant sterols or phytosterols (PS) are analogues of animal cholesterol in plants. They can be extracted from various by-products of pulp or paper industry or vegetable oil industry

(Moghadasian, 2000). Campesterol and sitosterol are formed by adding a methyl or ethyl group at carbon 24 of the cholesterol side chain (Fig. 2). The dehydrogenation of the carbon 22-23 bond yields stigmasterol. Hydrogenation leads to the formation campestanol and sitostanol.

The absorption of β-sitosterol in the alimentary canal of humans is about 5%, which is less than one sixth of the absorption rate of cholesterol, and the absorption of sitostanol is close to 0% (Heinemann et al., 1993). In rats the absorption of β-sitosterol is about 4%, and the absorption of sitostanol 1% (Sanders et al., 2000).

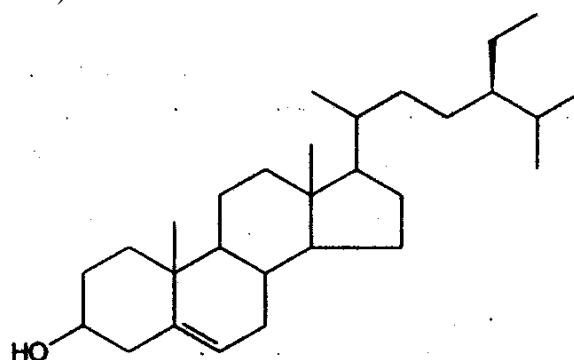


Fig. 2. The chemical structure of β-sitosterol.

In plasma, PS circulate carried by lipoproteins in rats (Boberg and Skrede, 1988) and humans (Miettinen, 1980). The plasma PS concentrations are usually 7-24 µmol l⁻¹, or less than 1% of total plasma sterols (Moghadasian, 2000). The daily intake of PS in the western diet is approximately 80 mg or more. In the Japanese or vegetarian diet, however, the daily PS intake can be as high as 400 mg.

PS accumulate in the liver, adrenals and gonads of rats (Sugano et al., 1978; Boberg et al., 1986; Sanders et al., 2000) – tissues secreting steroid hormones. PS can function as precursors of cortisol and sex steroids in humans and rats (Aringer et al., 1979; Moghadasian, 2000). Phytosterols are excreted mainly via bile into faeces in rats (Boberg et al., 1986; Sanders et al., 2000).

PS added into margarines are used to lower elevated serum cholesterol concentrations. β-Sitosterol at a dose of 2 g d⁻¹ decreases the total serum cholesterol significantly (Drexel et al.,

1981). At 1 g d⁻¹ it decreases cholesterol absorption by 42% (Mattson et al., 1982). Cholesterol absorption decreases to 39% with β -sitostanol, too (Normén et al., 2000).

There is extensive evidence on the beneficial effects that PS have on serum lipid levels. In rats PS lower the plasma cholesterol levels (Sugano et al., 1976, 1977; Malini and Vanithakumari, 1990). In hamsters a 0.5% supplement of β -sitosterol results in a 33% decrease in plasma total cholesterol (Smith et al., 2000). In gerbils, a two-month phytosterol treatment decreases plasma total and low density lipoprotein (LDL) cholesterol (Wasan et al., 2001a,b). In humans plant sterols or stanols at 2 g d⁻¹ (approximately 28 mg kg⁻¹ d⁻¹ for a 70 kg person) decrease plasma cholesterol equally by more than 10 % (Vanhanen et al., 1993; Miettinen et al., 1995; Gylling et al., 1997; Weststrate and Meijer, 1998; Hallikainen et al., 2000; Jones et al., 2000; Vissers et al., 2000; Neil et al., 2001). PS are effective even at lower doses with a reduction of 5-10% in total, LDL cholesterol or the LDL/high density lipoprotein (HDL) cholesterol ratio at a PS dose of about 1.6 g PS d⁻¹ (approximately 22 mg kg⁻¹ d⁻¹) (Hendriks et al., 1999) or even 0.8 g d⁻¹ (approximately 11 mg kg⁻¹ d⁻¹) (Sierksma et al., 1999).

As a result of decreased cholesterol absorption in the gut, the activity of 3-hydroxy-3-methylglutaryl CoA reductase (the rate-limiting enzyme in *de novo* cholesterol synthesis) increases in mice due to PS treatment (Moghadasian, 2000). Furthermore, in mice the lipoprotein and hepatic lipase activities decrease due to PS. Also in hamsters (Ntanios and Jones, 1999) and humans (Vanhanen et al., 1993) sitostanol causes an increase in cholesterol synthesis

β -Sitosterol has a protective influence against experimentally induced colon cancer in rats (Raicht et al., 1980). Human populations (e.g. vegetarians) with a lower cancer mortality than in the general population have in their diets a higher amount of PS in relation to cholesterol (Nair et al., 1984). β -Sitosterol may also protect against breast cancer (Awad and Fink, 2000). The growth of human prostate cancer cells can be inhibited with PS, as well (Awad et al.,

2000). β -Sitosterol can also have potential in the treatment of benign prostatic hyperplasia (Berges et al., 2000; Kassen et al., 2000).

There are also possible adverse effects of PS. β -Sitosterol inhibits the progesterone-induced acrosome reaction in human sperm (Khorasani et al., 2000). Sc administration of PS decreases the testicular weight and sperm count in albino rats at 0.5-5 mg kg⁻¹ d⁻¹ (Malini and Vanithakumari, 1991). Plant stanol esters cause an increase in absolute and relative testicular and epididymal weights as well as an increased number of lost implantations in rats (Whittaker et al., 1999). In female rats, β -sitosterol increases uterine glucose-6-phosphate dehydrogenase activity and uterine wet weight (Malini and Vanithakumari, 1992; 1993). This indicates to potential estrogenic effects of PS, which has not, however, been confirmed in other experiments (Baker et al., 1999; Turnbull et al., 1999).

Sitosterolemia is a rare autosomal recessive disorder characterized by increased dietary sitosterol absorption and reduced elimination (Bhattacharya and Connor, 1974; Miettinen, 1980). Symptoms include accelerated atherosclerosis, tendon xanthomas, arthritis, arthralgia and an early risk of acute myocardial infarction (Moghadasian, 2000).

PS enters the ecosystem e.g. via pulp mill effluents. *Chironomus riparius* (Diptera) larvae are relatively unaffected by β -sitosterol (Vermeulen et al., 2000). In the viviparous blenny (*Zoarces viviparus*), however, it has been observed that males exposed to PS at 10 μ g l⁻¹ have β -estradiol levels that are as high as those of females (Mattsson et al., 2001a). In prespawning goldfish (*Carassius auratus*), however, exposure to pulp mill effluents decreases testosterone levels in plasma and testes (McMaster et al., 1992). Furthermore, in fish, plasma sex steroid levels and gonad size are reduced (Van der Kraak et al., 1992). In the rainbow trout, β -sitosterol induces vitellogenin gene expression in the liver of juvenile and methyltestosterone-treated fish (Mellanen et al., 1996). β -Sitosterol *per se* decreases the 11-ketotestosterone concentrations of male and 17- β -estradiol concentrations of female goldfish (MacLatchy and Van der Kraak, 1994). As pulp

mill effluents, however, contain many other substances, it is possible that some of the effects are not due to PS only.

PS also stimulate the liver monooxygenase activity (ethoxyresorufin-*O*-deethylase or EROD) of rainbow trout at low (10 µg l⁻¹) and high (30 µg l⁻¹) concentrations but suppress it at intermediate concentrations (20 µg l⁻¹) (Mattsson et al., 2001b). In the blenny there is also an increase in larval deformities with increasing PS dose.

2.3 Endocrine and metabolic parameters

2.3.1 Leptin and ghrelin

Leptin is an adipocyte-derived hormone discovered by the positional cloning of the mouse *obese* (*ob*) gene (Zhang et al., 1994). Leptin is secreted principally by the white adipose tissue (Masuzaki et al., 1995; Tsuruo et al., 1996), the stomach (Bado et al., 1998), ovaries (Cioffi et al., 1997), mammary gland (Smith-Kirwin et al., 1998) and by many fetal tissues of the mouse (Hoggard et al., 1997).

In humans and rodents plasma leptin levels correlate significantly with body adiposity and body mass index (BMI=BM (kg) (length² or ³ (m))⁻¹). Obese individuals have higher plasma leptin concentrations than lean subjects (Maffei et al., 1995) and fasting in humans and laboratory rodents causes a rapid decline in the plasma leptin levels (Maffei et al., 1995; Considine et al., 1996; Kolaczynski et al., 1996). Leptin inhibits the secretion of neuropeptide Y (NPY) – a stimulator of feeding – in the hypothalamus (Stephens et al., 1995). Exogenous leptin reduces the food intake of genetically obese *ob/ob* mice and to a lesser degree of wild-type mice (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Also humans with congenital leptin-deficiency lose weight with leptin treatment (Farooqi et al., 1999). Leptin is needed to the maintenance of human menstrual cycle (Köpp et al., 1997) and to trigger the onset of puberty in many mammals (Aubert et al., 1997; Cheung et al., 1997; Strobel et al., 1998; Suter et al., 2000).

In carnivores and insectivores, however,

plasma leptin levels are often decoupled from body adiposity. This has been observed e.g. in the raccoon dog (*Nyctereutes procyonoides*), the blue fox (*Alopex lagopus*) (Nieminen et al., 2001, 2002), the mink (*Mustela vison*) (Nieminen et al., 2000), the common shrew (*Sorex araneus*) (Nieminen and Hyvärinen, 2000) and the Antarctic fur seal (*Arctocephalus gazella*) (Arnould et al., 2002). In nature leptin does not seem to be simply a feedback signal about the energy reserves of the body. Probably the falling leptin levels encountered in food deprivation (winter, drought etc.) initiate via the disinhibition of NPY the neuroendocrine response to fasting crucial for the survival of animals in nature (Ahima et al., 1996).

There are few studies on the effects of xenobiotics on plasma leptin concentrations. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has minor effects on rat leptin levels with an initial rise followed by falling leptin levels (Tuomisto et al. 1997). PCBs, however, have no effect on the plasma leptin concentrations of female minks (Nieminen et al., 2000).

Ghrelin is a novel signal peptide (Kojima et al., 1999). It is the endogenous analogue of different growth hormone secretagogues. Ghrelin is secreted by the stomach (Date et al., 2000) and hypothalamus (Kojima et al., 1999). Exogenous ghrelin increases food intake of rodents (Tschöp et al., 2000; Nakazato et al., 2001) and plasma ghrelin concentrations correlate inversely with body adiposity in humans (Tschöp et al., 2001).

In the hypothalamus ghrelin is antagonistic to leptin by stimulating the production of NPY, and the inhibition of NPY secretion by leptin can be blunted by ghrelin treatment (Shintani et al., 2001). In carnivores, however, leptin and ghrelin do not always seem to be antagonistic to each other (Nieminen et al., 2002).

2.3.2 Thyroid hormones

Thyroid tissue can be found in all vertebrates, in many protochordates and various ascidians (Bentley, 1998). The thyroid hormones tetraiodothyronine (T₄) and triiodothyronine (T₃) are formed from the amino acid tyrosine. T₃ is considered to be the effective hormone in

mammals (Despopoulos and Silbernagel, 1986). T_4 is converted into T_3 by a microsomal 5'-deiodinase. The plasma T_4 and T_3 concentrations are usually remarkably constant. Levels of these hormones are controlled by the adenohipophyseal thyroid-stimulating hormone (TSH). In mammals the amino acid sequence of TSH is 75-90 % identical between species (Bentley, 1998). T_3 and T_4 do not require specific receptor proteins. Instead, they are taken up by the target cells, bind to the DNA and influence transcription (Despopoulos and Silbernagel, 1986). T_3 and to a lesser degree T_4 stimulate energy turnover, growth, development of bones and the brain and increase heat production.

2.3.3 Steroid hormones

Steroid hormones are chemical compounds derived from cholesterol. Sex steroid hormones are very uniform in various phyla, and testosterone, estradiol and progesterone are identical in all vertebrates (Bentley, 1998). Testosterone promotes male sexual differentiation, formation of sperm and sexual drive (Despopoulos and Silbernagel, 1986). Estradiol promotes maturation of ovarian follicles and proliferation of uterine mucosa. Steroid hormones also penetrate the cell membrane easily, bind via a receptor complex to the DNA and influence transcription.

Sex steroid concentrations are regulated by hypophyseal hormones: follicle-stimulating hormone (FSH) and LH (Despopoulos and Silbernagel, 1986). In mammals LH regulates testosterone secretion via a negative feedback loop, and FSH stimulates the formation of an androgen-binding protein in Sertoli cells influencing spermatogenesis. In females FSH and LH participate in the regulation of the estrous cycle. Both these hormones stimulate the release of estradiol. In addition, LH initiates ovulation and the formation of *corpus luteum*.

2.3.4 Biotransformation enzymes

EROD is a specific enzyme to measure the activity of the cytochrome P450 (CYP1A) system (James, 1987). It belongs to the phase I

reactions that respond rapidly to various xenobiotics. EROD can be used as a sensitive indicator of the toxic burden of an organism (Stegeman and Lindström-Seppä, 1994). After the oxidative phase I reactions, the phase II reactions link the formed metabolites to endogenous water-soluble compounds.

The microsomal UDP-glucuronosyl-transferases (UDPGTs) catalyze the conjugation of foreign substances to glucuronic acid and the cytosolic glutathione S-transferases (GSTs) to glutathione (Armstrong, 1987). GSTs are able to form covalent bonds with products of phase I reactions and as a result prevent the binding of reactive xenobiotics to DNA.

2.3.5 Enzymes of lipid and carbohydrate metabolism

Glucose-6-phosphatase (G6Pase) is the final enzyme in the gluconeogenic pathway (Harris, 1986). The reaction catalyzed by G6Pase is irreversible in intracellular conditions. Thus G6Pase activity is a useful approximation of the the liberation of glucose into circulation. In the starve-feed cycle gluconeogenesis is important during periods of food shortage.

Glycogen phosphorylase activity, on the other hand, indicates the activity of glycogenolysis – glycogen degradation – the products of which are mostly used for intermediary metabolism instead of liberating glucose into circulation.

Hepatic lipases hydrolyze long-chain fatty acid glycerides and esterases short-chain fatty acid esters. Lipase esterase activity is a combination of the hydrolyzing activity of these enzymes in the liver.

2.3.6 Lipid parameters

Lipoproteins are a heterogenous group of protein-lipid complexes. They have crucial functions in lipid transport and metabolism (Schultz, 1986). Lipoproteins are classified according to their density. The lipoprotein classes are chylomicrons, very low density lipoproteins (VLDL), intermediate density

lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). The lipid fraction of lipoproteins consists of triacylglycerols, phospholipids, cholesterol, cholesterol esters and long-chain fatty acids. The most important protein components of lipoproteins are apolipoprotein A for HDL and apolipoprotein B100 for LDL.

HDL has an important role in reverse cholesterol transport, in which cholesterol is carried from peripheral tissues to the liver (Miller et al, 1985). LDL, on the other hand, carries cholesterol from the liver to the periphery, and it is involved in atherogenesis. There is a strong association between LDL cholesterol concentrations and coronary artery disease (Kannell et al., 1979) and the lowering of LDL cholesterol reduces morbidity and mortality in coronary disease (Gotto, 1995).

Triacylglycerols are compounds in which all three hydroxyl groups of glycerol are esterified with a fatty acid. Triacylglycerols constitute 99% of the lipid content of white adipose tissue and are the principal form of energy storage in many mammals (Van Pilsun, 1986). Triacylglycerols are hydrolyzed in fasting by lipases (Harris, 1986). Hypertriglyceridemia with low HDL cholesterol concentrations is also considered to be a cardiovascular risk factor (Brunzell, 1988).

2.4 Experimental animals

2.4.1 The European polecat

The European polecat (*Mustela putorius* L. 1758, Mustelidae, Carnivora) (Fig. 3) is a mustelid carnivore. The ferret (*Mustela putorius furo*) is the semi-domesticated subspecies used more frequently as an experimental animal. The body of the polecat is elongated, and the body length is approximately 40 cm without tail. The polecat is sexually dimorphic: the male (BM 1.0–1.5 kg in nature) is considerably heavier than the female (BM 0.6–0.8 kg in nature) (Wolsan, 1993). The species is fairly common in Southern and Central Finland and Central Europe. It lives especially on forest edge but also near human habitation. The polecat is a

true carnivore with a diet consisting mostly of frogs, rodents, birds, insects and cadavers. Yet some plant items can be included in its diet e.g. the fruits of the prune (*Prunus domestica*), the vine (*Vitis vinifera*) and the apple (*Malus domestica*).

The reproductive season of the polecat is between March and June (Björvall and Ullström, 1995). Unlike some other mustelids no delayed implantation is encountered. The female gives birth to 5-10 cubs after a gestation period of 40-42 days.



Fig. 3. The European polecat (*Mustela putorius*).

The polecat is a typical long-day mammal with its reproductive period closely associated with day length. Renewed gonadal function and LH secretion can be observed when daylight is equal or in excess of 8 hours of light and 16 hours of darkness (Ryan, 1985; Jallageas et al., 1994). Female polecats can be brought to estrus by extension of the photoperiod from 8 to 16 h daily (Donovan et al., 1983). At the onset of estrus a rise in plasma estradiol levels can be observed together with falling blood levels of testosterone, progesterone and FSH. In intact anestrus female polecats LH is secreted episodically in pulses occurring at a frequency of 0.4 pulses h⁻¹ (Ryan et al., 2000). Removal of ovaries causes an increase in LH secretion in a typical mammalian feedback pattern. Estrogen exerts a negative feedback on the LH secretion of both females and males (Carroll and Baum, 1989).

2.4.2 The field vole

The field vole (*Microtus agrestis* L. 1761, Arvicolidae, Rodentia) (Fig. 4) is a small rodent species common in both Scandinavia and Central Europe (Björvall and Ullström, 1995). It lives principally on open grassland, cultivated land or alpine meadows. The BM of an adult male field vole is approximately 40 g and the BM of a female 35 g (Krapp and Niethammer, 1982). The species reproduces between April and September as a seasonal breeder influenced by the photoperiod. After a 20-day pregnancy the female gives birth to a litter of up to five individuals, and it can be fertilized again almost immediately (Myllymäki, 1977). The animals reach sexual maturity at the age of 50-60 days.



Fig. 4. The field vole (*Microtus agrestis*).

In the female field voles ovulation depends on external stimuli, such as male-female interactions and copulation (Chitty and Austin, 1957; Breed 1967). Female microtines paired with males exhibit estrous cycles of 4-5 days, unpaired females are anestrus. This phenomenon is called induced ovulation. Mean plasma concentrations of progesterone and testosterone of unpaired females are significantly lower than in paired females (Nubbemeyer, 1999). Mated female field voles experience a rapid and marked elevation of plasma LH concentrations (Milligan, 1981). This LH surge cannot be induced by exogenous steroids, such as 17- β -estradiol, estradiol benzoate or progesterone (Milligan, 1978).

3 AIMS OF THE STUDY

The aim of the present studies was to observe *in vivo* the effects that BPA and PS could have on the endocrinology, biotransformation and lipid and carbohydrate metabolism of mammals. Previously, endocrine disruptors have been studied extensively in aquatic invertebrates and vertebrates and laboratory rodents (rats and mice). BPA was chosen to represent widely-used synthetic compounds and PS to represent natural compounds. Humans encounter both BPA and PS in daily life. BPA may enter the human body from e.g. food packaging and PS from various cholesterol-lowering spreads. Thus the potential risks of these compounds to nature and human populations are significant.

Two animal species with different life histories were chosen to compare the effects of endocrine disruptors on carnivores and herbivores. As PS are produced by various plants, it is conceivable that herbivores could have developed better adaptations than carnivores to the possible deleterious effects of PS. The polecat was chosen to these studies to represent a fairly common carnivore. As top predators, mustelids, such as the polecat and the mink are very susceptible to various xenobiotics (Aulerich et al., 1987; Shipp et al., 1998) making them attractive models and possible bioindicator species. The field vole represents a common small herbivore. As a common and fairly easily accessible species, it also has potential as a bioindicator species.

Several biochemical parameters were measured. Previous studies have mainly been conducted either *in vitro* or they have mostly concentrated on only a few parameters (sex steroids, serum lipids). In contrast, the studies of this thesis aimed to screen different parameters as possible biomarkers for risk assessment and environmental monitoring. As all the exposures were subacute with a relatively small number of experimental animals, these studies must also be taken only as preliminary experiments aimed to distinguish the most susceptible physiological parameters to be measured in future studies with chronic exposure.

The specific aims were as follows:

1. What are the effects of BPA and PS on plasma testosterone, estradiol and gonadotropin concentrations of carnivorous and herbivorous mammals (I-IV)?
2. Are there other endocrine effects of BPA and PS (I-IV)?
3. How do BPA and PS affect the biotransformation apparatus of the selected species (I-IV)?
4. What are the effects of PS on lipid and carbohydrate metabolism and weight regulation of carnivores and herbivores (III-IV)?
5. Are there suitable biomarkers for risk assessment or environmental monitoring for the effects of BPA or PS (I-IV)?

4 MATERIALS AND METHODS

4.1 Experimental animals and study designs

To study the effects of BPA (I), 40 pre-breeding European polecats (20 males and 20 females) were randomly assigned into four study groups. The randomizations yielded experimental groups with no differences in the initial BM between groups in all the studies (I-IV). The polecats (I) were kept at a fur farm (Liperi, Finland) in individual cages in shadowhouses under roof but exposed to ambient temperature and photoperiod. The animals were exposed to peroral BPA for two weeks between January 31th and February 14th 2000. The control group was fed with a commercial fur animal feed (33% protein, 24% fat, 2.3% fiber, 7.5% ash, 0.4% calcium, 16 000 IU vitamin A kg⁻¹ dry matter, 1400 IU cholecalciferol kg⁻¹ dry matter) The males received about 250 g (1410 kJ) and the females 200 g (1130 kJ) feed d⁻¹. The other three groups were fed with the same amount of the same feed with BPA mixed into the feed at doses of 10, 50 or 250 mg kg⁻¹ d⁻¹.

The BM of the animals was measured at the beginning of the experiment and thereafter at one-week intervals. At the end of the study, the body length from the tip of the nose to the anus was measured. From these data the BMI was calculated.

For the PS experiment (III) 32 juvenile prebreeding polecats (16 males and 16 females) were randomly assigned into four study groups. Housing and feeding of the animals was identical to study I. The study period was between November 27th and December 11th 2000. The first of the study groups was the control group and the other groups were fed with Ultra-Sitosterol (88.7% β -sitosterol and β -sitostanol, 9.0% campesterol and campestanol, 0.9% artenols; UPM Kymmene, Kaukas, Lappeenranta, Finland) for two weeks mixed into the daily feed of the animals at 1, 5 or 50 mg kg⁻¹ d⁻¹. BM and length were measured as in study I.

To study the effects of BPA on the field vole (II), 48 field voles (23 males and 25 females) were randomly assigned into four study groups. The animals were from the breeding colony of the University of Joensuu and of the F1 generation of parents captured in the wild (Punkaharju, Finland). All the animals were 3-5 months old at the beginning of the experiment and thus sexually mature. The voles were marked with a felt pen and housed in groups of 3 animals of the same litter and sex but of different study groups in solid bottom plastic cages (Makrolon: 42 x 22 x 15 cm). The voles had wood shavings for bedding and free access to water and a pelleted diet (Avelsfåder för råtta och mus R36 containing 18.5% protein, 4.0% fat with an energy content of 1260 kJ 100 g⁻¹, Lactamin, Stockholm, Sweden). The animals were weighed at the beginning and the end of the study period.

The study period (II) was between November 14th and November 18th 2000. The first group (6 males, 5 females) was the control group. The second group (4 males, 7 females) received BPA at 10 mg kg⁻¹ d⁻¹, the third group (6 males, 5 females) at 50 mg kg⁻¹ d⁻¹ and the fourth group (7 males, 8 females) at 250 mg kg⁻¹ d⁻¹. BPA flakes were dissolved into propylene glycol according to Hanioka et al.

(1998) in solutions of 20 mg ml⁻¹, 75 mg ml⁻¹ and 500 mg ml⁻¹. BPA solution was injected daily into the loose interscapular sc tissue with sterile needles and syringes. The volume of a single BPA-propylene glycol injection was approximately 15 µl in all dose groups. The control group received daily injections of 15 µl of propylene glycol only.

31 field voles (14 males and 17 females) were chosen for the PS study (IV) and assigned into three study groups. The animals were from the same breeding colony as the voles in study II. The animals were housed singly in plastic cages. Otherwise the housing, weighing and feeding of the animals was identical to study II.

The study period (IV) was between May 9th and May 23rd 2001. The first group (3 males and 4 females) was the control group. The second group (4 males and 7 females) received Ultra-Sitosterol (Study III) perorally mixed into the normal feed of the animals at 5 mg kg⁻¹ d⁻¹ and the third group (7 males, 6 females) at 50 mg kg⁻¹ d⁻¹. Food intake was recorded by weighing of the uneaten food at the end of the study. The Animal Care and Use Committee of the Department of Biology, University of Joensuu approved all the procedures on the experimental animals.

4.2 Sample collection and storage

The polecats (I, III) were sacrificed at the end of the study periods with an electric shock that killed the animals immediately. Blood samples were obtained with cardiac punctures into test tubes containing EDTA to prevent clotting for the plasma hormone determinations (I, III) and into plain test tubes for the serum lipid determinations (III). The EDTA tubes were centrifuged immediately at 1000 g to obtain plasma; the serum samples were left to stand for 30 minutes, after which they were also centrifuged at 1000 g. The livers, kidneys and testes of the animals were dissected and all the samples were frozen in liquid nitrogen, and stored at -40°C for the hormone determinations and measurements of enzyme activities of lipid and carbohydrate metabolism, and at -80°C for the measurement of biotransformation enzyme

activities.

The field voles (II, IV) were sacrificed at the end of their study periods with diethyl ether. They were weighed, their lengths were measured and sex confirmed intra-abdominally. Blood samples were obtained with cardiac punctures into test tubes containing EDTA and centrifuged immediately at 4000 g to obtain plasma. Approximately 200-500 µl blood was obtained per animal yielding about 75-200 µl plasma. The livers and kidneys of the voles were dissected and all the samples were frozen in liquid nitrogen. The samples were stored at -40°C for the hormone determinations and measurements of enzyme activities of lipid and carbohydrate metabolism, and at -80°C for the measurement of biotransformation enzyme activities.

4.3 Biochemical measurements

4.3.1 Hormone assays

Hormone concentrations were measured using radioimmunoassay (RIA), immunoradiometry (IRMA) and immunoassay (ELISA) methods. Plasma testosterone (II-IV), estradiol (I-III), cortisol (I-III), T₄ (II-IV) and T₃ (III) concentrations were measured with the Spectria [¹²⁵I] Coated Tube Radioimmunoassay kits (Orion Diagnostica, Espoo, Finland). Plasma TSH (II-IV) and LH (II-IV) concentrations were measured with IRMA [¹²⁵I] Coated Tube Immunoradiometric Assay kits of Orion Diagnostica. For study I the T₄ and T₃ concentrations were determined using the Canine T₄ and T₃ kits (Diagnostic Products Corporation (DPC), Los Angeles, Ca, USA), the FSH concentrations with the FSH Double Antibody kit (DPC) and the TSH levels with the Canine TSH IRMA (DPC). In study IV the estradiol concentrations were measured using the immunoassay method (17 β-estradiol Immuno-assay, R&D Systems, Wiesbaden-Nordenstadt, Germany).

Plasma leptin concentrations (I-IV) were determined with the Multi-Species Leptin RIA kit (Linco Research, St Charles, MO, USA) and the plasma ghrelin concentrations (II-IV) using the Ghrelin (Human) RIA kit (Phoenix

Pharmaceuticals, Belmont, CA, USA). The leptin and ghrelin assays were validated with dilution series. For the actual measurements a gamma counter (1479 Wizard, Wallac, Turku, Finland) was used.

The plasma leptin, ghrelin, LH, TSH, cortisol (II, IV) and estradiol (II) concentrations of the voles were measured by pooling 5-10 μ l plasma of individual animals of the same study group together as there was an insufficient amount of plasma to measure these hormone concentrations from individual animals.

4.3.2 Enzymatic analyses

To measure the liver and kidney glycogen content and the activities of enzymes of carbohydrate and lipid metabolism (II-IV) the liver and kidney samples were weighed and homogenized in cold citrate buffer, pH 6.5 for the G6Pase and pH 6.1 for the glycogen phosphorylase measurements. For the lipase esterase measurement, the homogenization was carried out in cold 0.85% sodium chloride. G6Pase activity was measured using glucose-6-phosphate as substrate in the presence of EDTA after a 30 min incubation at 37.5°C (Hers and van Hoof, 1966). Glycogen phosphorylase activity was measured in the presence of glycogen, glucose-1-phosphate, sodium fluoride and AMP according to the method of Hers and van Hoof (1966). Lipase esterase activity was measured using 2-naphtyl laurate without taurocholate as substrate (Seligman and Nachlas, 1962). The liver and kidney glycogen concentrations were measured according to the method of Lo et al. (1970). All these measurements were carried out with the Hitachi U-2000 Spectrophotometer (Tokyo, Japan).

For the preparation of microsomes to measure the biotransformation enzyme activities the thawed liver (I-IV) and kidney (II-IV) samples were homogenized in ice-cold 0.25 M sucrose, pH 7.4. The homogenized samples were centrifuged at 10 000 g for 20 min, and the supernatants were subsequently centrifuged at 105 000 g for 60 min to pellet the microsomes. The pellets were resuspended in 0.25 M sucrose to a volume of 1 ml g^{-1} tissue.

The hepatic monooxygenase activity was

measured according to the method of Burke and Mayer (1974) with the Shimadzu spectrophotometer (RF-5001PC) with resorufin as the internal standard. The UDPGT activity was measured with Shimadzu UV-240 Spectrophotometer in an incubation mixture containing 0.35 mM *p*-nitrophenol as aglycone and 4.5 mM UDP-glucuronic acid in the presence of 20 mM K_2EDTA (Hänninen, 1968).

The cytosolic GST activity was measured according to the method of Habig et al. (1974) with 1-chloro-2,4-dinitrobenzene as substrate in a Perkin-Elmer Lambda 2 UV/VIS spectrophotometer at 340 nm. All these measurements were carried out at 37°C and controlled to be linear with time and enzyme concentration. The protein content of the microsomal and supernatant fractions were measured according to the method of Bradford (1976).

4.3.3 Lipid analyses

Serum lipids (III) were measured at the LabHolding Laboratory (Tampere, Finland). The serum total cholesterol concentrations were measured spectrophotometrically (Burtis and Ashwood, 1996) at 510 nm wavelength (Konelab 60 I, Thermo Labsystems CLD, Espoo, Finland).

The serum HDL concentrations were also measured spectrophotometrically (Assmann et al., 1983; Burtis and Ashwood, 1996; Thomas, 1998). The peroxidase reaction yielded a purple-blue pigment measured at 510 nm. For the measurement of the serum triglyceride concentrations the triglycerides in the serum were hydrolyzed by lipase to glycerol and fatty acids. Glycerol was subsequently phosphorylated to glycerol-3-phosphate, which was oxidized to dihydroxyacetone phosphate and hydrogen peroxide. Hydrogen peroxide reacted with 4-aminoantipyrine and 4-chlorophenole to form a quinoneimine dye, the absorbance of which was measured at 510 nm (Burtis and Ashwood, 1996). The serum LDL concentrations were calculated by the formula: $S\text{-LDL} = S\text{-total cholesterol} - S\text{-HDL} - (S\text{-triglycerides}/2.2)$.

4.4 Statistical analyses

Multiple comparisons were performed with the one-way analysis of variance (ANOVA) followed by the *post hoc* Duncan's test. The $p < 0.05$ level was considered to be statistically significant. Paired comparisons – for instance comparisons between the sexes – were performed with the Student's t-test. The normality of distribution and the homogeneity of variances were determined with the Kolmogorov-Smirnov test and with the Levene test. For nonparametric data the Mann-Whitney U-test was used (for example plasma testosterone). To analyse mortality in study III the χ^2 -test was also used.

Correlations were calculated using Spearman's rank correlation coefficient (r_s). In studies I and II also a discriminant analysis was performed to express differences between the study groups. The results are expressed as the mean \pm SE (II-IV) or as the mean \pm SD (I). If there was no sexual dimorphism in a measured parameter, the results of males and females were analyzed and presented together.

5 RESULTS

5.1 General parameters

All the polecats (I, III) and the field voles exposed to PS (IV) remained at good health throughout the studies. No macroscopic effects were observed in the well-being of the experimental animals or in the organs at necropsies. PS at $50 \text{ mg kg}^{-1} \text{ d}^{-1}$ increased the food intake of the field voles significantly (Mann-Whitney U test, $p < 0.003$) without any effect on the BM of the voles.

There was, however, significant mortality in the field voles exposed to BPA (II, Table 2). The mortality was 0% in the control voles, 18.2% at $10 \text{ mg kg}^{-1} \text{ d}^{-1}$, 36.4% at $50 \text{ mg kg}^{-1} \text{ d}^{-1}$, and 20.0% at $250 \text{ mg kg}^{-1} \text{ d}^{-1}$ (χ^2 test, $p < 0.05$). BPA or PS did not cause any differences in the BM or BMIs of the experimental animals. As both these species are dimorphic, the BM and absolute liver and kidney weights of the males were higher than those of the females (I-IV).

In female polecats BPA caused an increase in the absolute and relative liver weights at $250 \text{ mg kg}^{-1} \text{ d}^{-1}$ (I, Table 1). There were no differences in the absolute or relative liver weights in polecats exposed to PS (III) or in field voles exposed to BPA (II), but the relative liver weights of the voles treated with PS decreased significantly when the PS exposed groups together were compared to the control animals (t-test, $p < 0.05$) (IV).

The absolute or relative testicular weights of the males were not affected by either BPA or PS treatments (I-IV). In the field voles treated with PS (IV), however, the mean testicular weight was the highest at $5 \text{ mg kg}^{-1} \text{ d}^{-1}$, and the difference to the control group was nearly significant (t-test, $p < 0.06$).

Table 1. Principal effects of BPA on the polecat. The animals were exposed to BPA perorally for two weeks.

-
- Relative liver mass elevated in females
 - Slight increase in plasma testosterone concentrations
 - Liver UDPGT and GST activities increase slightly, especially in females
-

Table 2. Principal effects of BPA on the field vole. The animals were exposed to BPA subcutaneously for four days.

-
- Increased mortality
 - Increase in plasma testosterone concentrations
 - Pooled eptin concentration increased and pooled ghrelin concentration decreased
 - Liver EROD and GST activities suppressed
-

5.2 Effects on reproductive hormones

In the polecats exposed to BPA no significant effects could be seen in the plasma estradiol or FSH concentrations (I). The testosterone levels did not differ between the experimental groups, either, but the testosterone concentrations of the male polecats increased slightly with increasing

BPA dose and correlated positively with the BPA dose ($r_s = 0.381$, $p < 0.05$, Table 1).

In the field voles, plasma estradiol concentrations measured from pooled plasma were lower in the BPA-exposed groups than in the control group. The individual plasma testosterone concentrations increased at 250 mg BPA $\text{kg}^{-1} \text{d}^{-1}$ in both sexes (Mann-Whitney U test, $p < 0.023$, II, Table 2). At the same time, the LH levels measured from pooled plasma increased.

In the PS experiments the plasma testosterone concentrations of the polecats (III) correlated positively with increasing PS dose when males and females were analyzed together ($r_s = 0.894$, $p < 0.01$, Table 3). The estradiol levels of male polecats increased at 50 mg PS $\text{kg}^{-1} \text{d}^{-1}$ compared to control males (t-test, $p < 0.05$), and the female polecats exposed to 5 or 50 mg PS $\text{kg}^{-1} \text{d}^{-1}$ had higher plasma estradiol concentrations than the control females (t-test, $p < 0.05$). In the polecats there were no differences in the plasma LH concentrations between the experimental groups.

In the field voles PS caused biphasic responses in the sex steroid levels (IV, Table 4). The plasma testosterone concentrations were slightly higher at 5 mg PS $\text{kg}^{-1} \text{d}^{-1}$ compared to the other groups but the difference was not significant due to high intergroup variance. The plasma estradiol concentrations were the highest at 5 mg PS $\text{kg}^{-1} \text{d}^{-1}$ compared to the control group or the 50 mg PS $\text{kg}^{-1} \text{d}^{-1}$ group (ANOVA, $p < 0.05$). The plasma LH concentration measured from pooled plasma was the lowest at 5 mg PS $\text{kg}^{-1} \text{d}^{-1}$.

Table 3. Principal effects of PS on the polecat. The animals were treated with peroral phytoesterol-stanol mixture for two weeks.

-
- Plasma estradiol and testosterone concentrations increase
 - Ghrelin concentrations decrease
 - Increase in liver glycogen content
 - Increase in liver G6Pase activity
 - Decrease in liver lipase esterase activity
 - LDL cholesterol levels increase
-

Table 4. Principal effects of PS on the field vole. The animals were treated with peroral phytoesterol-stanol mixture for two weeks.

-
- Increase in food intake
 - Biphasic responses in plasma estradiol, LH, leptin and ghrelin concentrations and liver G6Pase activity
 - Biotransformation enzymes unaffected
-

5.3 Thyroid hormones and cortisol

BPA did not affect the T_4 , T_3 or TSH concentrations of the polecats (I). Nor were there any differences in the T_3 - T_4 ratio. The T_4 levels of the field voles were not affected by BPA, either (II). The T_4 concentrations of the male field voles in the BPA experiment were, however, higher than the concentrations of the females (t-test, $p < 0.002$). The TSH levels measured from pooled plasma did not differ between the experimental groups.

PS caused an increase in the plasma T_4 concentrations of the polecats at 5 or 50 mg PS $\text{kg}^{-1} \text{d}^{-1}$ compared to the control group and the 1 mg PS $\text{kg}^{-1} \text{d}^{-1}$ group together (t-test, $p < 0.02$) (III). The T_3 - T_4 ratio was higher at 1 or 50 mg PS $\text{kg}^{-1} \text{d}^{-1}$ (ANOVA, $p < 0.05$). There were no differences in the plasma TSH concentrations due to PS.

In the field voles no differences could be seen in the plasma T_4 concentrations due to PS (IV). The TSH values measured from pooled plasma were below the detection limit in the PS exposed groups, but in the control group the TSH value was clearly higher (0.443 mIU ml^{-1}).

The plasma cortisol concentrations of the polecats treated with PS did not differ between the experimental groups (III), and the plasma cortisol values measured from pooled plasma of the BPA-exposed field voles were below the detection limit in all groups (II). In the BPA-exposed polecats (I), however, the control females had the highest plasma cortisol concentrations, and the difference between the control females and the 50 mg BPA $\text{kg}^{-1} \text{d}^{-1}$ group was significant (t-test, $p < 0.05$).

5.4 Leptin and ghrelin

The plasma leptin concentrations of the polecats did not change either due to BPA or PS (I, III). The plasma ghrelin concentrations of the PS-treated polecats were significantly lower at 50 mg PS kg⁻¹ d⁻¹ (ANOVA, $p < 0.05$, III, Table 3). In the PS study the plasma leptin levels of the polecats correlated significantly with the BMIs ($r_s = 0.523$, $p < 0.01$).

In the field voles, the plasma leptin value measured from pooled plasma of the BPA-exposed voles decreased at higher doses and, at the same time, the ghrelin value measured from pooled plasma increased slightly (II). In the PS treated voles (IV, Table 4), the leptin value decreased at 5 mg PS kg⁻¹ d⁻¹ and, at the same time, the ghrelin value increased with a return to the levels of the control animals at 50 mg PS kg⁻¹ d⁻¹.

5.5 Biotransformation enzymes

BPA increased the liver GST activity of the female polecats (I) at 250 mg BPA kg⁻¹ d⁻¹ (ANOVA, $p < 0.05$). The liver UDPGT activity of the females (ANOVA, $p < 0.05$) and of the males (t-test, $p < 0.05$) increased as well at 250 mg BPA kg⁻¹ d⁻¹ (I). The UDPGT ($r_s = 0.475$, $p < 0.05$) and the GST ($r_s = 0.428$, $p < 0.05$) activities correlated with BPA dose (Table 1).

In the field voles, the liver EROD activity decreased at all BPA doses (II, Table 2) (t-test, $p < 0.04$). Liver GST activity decreased at 250 mg BPA kg⁻¹ d⁻¹ compared to the control group (t-test, $p < 0.03$). There were no differences in the UDPGT activity or the kidney EROD or GST activities.

In the PS-treated polecats (III) there was some fluctuation in the liver EROD activity with the highest activity at 1 mg PS kg⁻¹ d⁻¹ (ANOVA, $p < 0.01$), and the females had higher EROD and GST activities in the liver than the males (ANOVA, $p < 0.05$). In the kidneys the GST activity was the highest at 50 mg PS kg⁻¹

d⁻¹ (ANOVA, $p < 0.05$). In the field voles PS had no effect on the biotransformation enzyme activities in the liver (IV).

5.6 Carbohydrate and lipid metabolism

In the polecats, PS treatment increased the liver glycogen content at 50 mg PS kg⁻¹ d⁻¹ (ANOVA, $p < 0.05$, III, Table 3). In the females the increase was twofold, but in the males almost fourfold. G6Pase or phosphorylase activities in the livers were not affected, but the liver lipase esterase activity decreased at 5 or 50 mg PS kg⁻¹ d⁻¹ (ANOVA, $p < 0.05$).

In the kidneys, the G6Pase activity increased at 50 mg PS kg⁻¹ d⁻¹ (ANOVA, $p < 0.05$) and the kidney glycogen phosphorylase activity increased in the females at 1 mg and 50 mg PS kg⁻¹ d⁻¹ (ANOVA, $p < 0.05$) (III). The liver lipase esterase activity of the polecats correlated negatively with the serum total cholesterol concentrations ($r_s = -0.405$, $p < 0.05$).

The serum total cholesterol, HDL cholesterol or the triglyceride concentrations of the polecats did not differ, but the serum LDL cholesterol concentrations increased at 50 mg PS kg⁻¹ d⁻¹ (ANOVA, $p < 0.05$; Table 3). At the same time, the HDL/cholesterol ratio decreased (ANOVA, $p < 0.05$). The HDL cholesterol levels of the polecats were very high (about 5.1 mmol l⁻¹) and the LDL cholesterol levels very low (0.44-1.00 mmol l⁻¹) compared to human values.

In the field voles the effects of PS on the carbohydrate and lipid metabolism were, again, biphasic (IV). The liver G6Pase activity and the glycogen phosphorylase activity were the highest at 5 mg PS kg⁻¹ d⁻¹ (ANOVA, $p < 0.05$; Table 4). The kidney glycogen phosphorylase activity, however, decreased in the PS treated groups (ANOVA, $p < 0.05$). The liver or kidney glycogen content or the liver lipase esterase activities were not affected.

Table 5. Summary of the results of studies I-IV. ↑/↓ = Statistically significant increase/decrease in the measured parameters. (↑)/(↓) = Statistically significant correlation or nonsignificant trend, or a difference in measurements from pooled plasma, nd = not determined, 0 = no change in the measured parameter. L = liver. Bold typeface is used to highlight similar changes or results in different studies.

	Polecat and BPA	Field vole and BPA	Polecat and PS	Field vole and PS
BM	0	0	0	0
BMI	0	0	0	0
Food intake	nd	nd	0	↑
Relative liver mass	↑	0	0	↓
Testicular mass	(↑)	0	0	(↑) at 50 mg
Estradiol	0	0	↑	↑ at 5 mg
Testosterone	↑	↑	(↑)	↑
LH	nd	(↑)	0	↓ at 5 mg
FSH	0	nd	nd	nd
T ₄	(↑)	0	↑ in males	0
T ₃	0	0	0	0
TSH	0	0	0	(↓)
Leptin	0	(↓)	(↓)	(↑) at 5 mg
Ghrelin	nd	(↑)	↓	(↓) at 5 mg
L glycogen	nd	nd	↑	0
L G6Pase	nd	nd	↑	↑ at 5 mg
L Lipase esterase	nd	nd	↓	0
L Phosphorylase	nd	nd	0	0
L EROD	0	↓	0	0
L UDPGT	↑	0	0	0
L GST	↑ in females	↓	0	0
Cholesterol	nd	nd	0	nd
LDL	nd	nd	↑	nd
HDL	nd	nd	0	nd

6 DISCUSSION

6.1 General remarks

These studies were carried out on experimental species not previously used to measure the possible effects of BPA or PS. As both the species are terrestrial, they could have potential as indicators of endocrine disruption in terrestrial ecosystems. The great number of measured parameters yielded many effects not observed or measured previously. In some cases, the amount of data with many marginally significant effects made the results puzzling and even contradictory.

The interpretation of the results is also complicated by the fact, that the experiments were carried out on a relatively small number of animals. In study II (field voles exposed to BPA) this was further jeopardized by the high mortality diminishing the number of animals available for sampling. In addition, as the BPA or PS dose increased (especially II-IV), so did the interindividual variation in the measured parameters. In this context, some of the observed effects can be due to methodological limitations: fluctuations caused by the small number of experimental animals and variable results – not real effects caused by the substances.

The effects of BPA seemed to be more pronounced on the field vole compared to the polecat (Table 1-2, 5) with e.g. increased mortality. In this case, comparison between the two species is, however, very difficult due to the sc administration route of BPA in the field vole, as the bioavailability of BPA is significantly higher if administered sc compared to peroral treatment (Laws and Carey, 1997; Yoo et al., 2001). Due to this it is understandable that also the effects would be more pronounced on the vole. Also the shorter study period (four days) of study II compared to the other studies makes the comparison of effects between the two different compounds and species more difficult. Yet the effects of BPA on the field vole do offer new information compared to laboratory rodents (rats) as similar experiments with a similar time period have been carried out previously in the

rat (Hanioka et al., 1998).

The PS fed to the polecats and field voles was a mixture of β -sitosterol, -sitostanol and, to a lesser degree other PS (campesterol, campestanol, arstenols). Due to this it is impossible to know with certainty, whether the effects were caused by β -sitosterol, β -sitostanol or a combination of these molecules. As the absorption of β -sitosterol in the intestine is, however, much higher than the absorption of stanols (Heinemann et al., 1993; Sanders et al., 2000), it is conceivable that β -sitosterol would be the principal causative agent of the observed effects. Furthermore, animals and humans consume many different PS as a part of their diets. A mixture of PS is also encountered in pulp mill leachates. Thus the Ultra-Sitosterol mixture used in these experiments could be closer to the substances encountered either in nature or in the daily diet of the general population than the more purified products would be. It does, however, make the results less useful from the point of view of human risk assessment.

Measurement of hormone concentrations from pooled plasma was a necessity in the case of the field voles, as the amount of plasma obtained would have been insufficient for measuring all the endocrine parameters from each individual vole. Thus, in these cases, no statistical analyses were possible, and the results of these measurements must be taken as preliminary and they need to be reconfirmed in future studies concentrating on selected hormone levels of individual animals. Also the fact that the pooling was performed using mixed plasma of both female and male voles of the same dose group complicates the interpretation of the results. In these cases (II, IV), it would have been more prudent to pool plasma using only plasma of one sex if enough plasma had been obtained from the sampling. Due to this, too, the results of these hormones (leptin, ghrelin, hypophyseal hormones, estradiol in study II) must be taken as preliminary. Yet they can provide startpoints to chronic exposure studies.

In hindsight, it would have been useful to measure identical parameters in each of these

studies. For instance, at the beginning of these experiments, no effects of BPA on the liver carbohydrate or lipid metabolism were determined, and thus these possible effects remained uninvestigated. In the case of ghrelin (absent from study I), the information about the discovery of this hormone unfortunately did not reach us in time (Kojima et al., 1999). Finally, it would have been very interesting to see how PS would have affected the serum total and LDL cholesterol concentrations of the field voles, but due to the small amount of blood obtained from the voles some analyses had to be prioritized. In these studies, sex steroids and thyroid hormones were given priority.

As a result of these methodological limitations, comparisons between different exposures and species could not be made in all cases, and the emphasis of these comparisons had to be on the effects on the endocrine and biotransformation parameters.

6.2 The effects of BPA

6.2.1 General parameters

In the polecat, the effects of BPA were relatively few (Table 1), but the discriminant analysis, however, classified the animals at 50 or 250 mg BPA kg⁻¹ d⁻¹ apart from the other two experimental groups indicating a deviation from the physiology of the control group at a hypothetical BPA dose between 10 and 50 mg kg⁻¹ d⁻¹. The relatively small number of experimental animals (40) could, of course, have masked possible differences in hormone parameters (e.g. testosterone) that, however, showed a linear response with increasing exposure. No macroscopic effects could be seen in the well-being of the animals indicating to the only mild or moderate acute toxicity of BPA (Alexander et al., 1988).

The absolute and relative liver weights of the female polecats increased at a high BPA dose (I). This fits to the observation of the higher GST and UDPGT activities in the females at 250 mg BPA kg⁻¹ d⁻¹. The activation of these enzymes could be connected to the increase in absolute and relative liver weights. In the

polecat, there was also a clear and expected sexual dimorphism in the BM, absolute liver weight and length of the animals. As the polecat is a dimorphic species it is understandable that also the morphological and physiological parameters of the sexes should differ (see Mustonen et al., 2000).

There was significant mortality in the BPA-exposed field voles (Table 3). Due to the sc administration route this effect cannot be compared to the effects of BPA on the polecats, as the bioavailability of BPA is higher when administered sc (Laws and Carey, 1997). Thus it is understandable that the voles perished, when exposed to BPA sc at a dose that was virtually ineffective when administered to the polecats perorally. Yet the results of this study (II) indicate to higher susceptibility of the field voles to the toxic effects of s.c. BPA compared to e.g. rats (see e.g. Hanioka et al., 1998). The toxicity of BPA is targeted at multiple organ systems: kidneys, liver, spleen, pancreas and lungs (Atkinson and Roy, 1995a,b; Yoo et al., 2000). Mitochondrial respiration is also affected (Nakagawa and Tayama, 2000). It seems possible that these effects are more pronounced in the vole causing the observed high mortality. Yet no direct cause of death could be seen in the autopsies, and the specific targets of BPA toxicity to the field voles will have to be determined in the future.

6.2.2 Endocrine effects

BPA is supposed to have estrogenic activity (Krishnan et al., 1993, Nagel et al., 1997). In the present studies, however, no clear effect on the sex steroids of the polecat could be seen due to BPA exposure (I). The testosterone levels of male polecats, however, correlated with the increasing BPA dose.

In the field voles (II) there was an increase in the individual plasma testosterone concentrations and the plasma estradiol concentrations measured from pooled plasma. At the same time the LH values of the field voles measured from pooled plasma increased. This effect is different from previous results on the male rat (Tohei et al., 2001) with a decrease in plasma testosterone concentrations and an

increase in the plasma LH concentrations due to BPA. This result is puzzling. Normally field voles experience a rapid and marked elevation of plasma LH levels immediately after mating (Milligan, 1981). No behavioral induction of LH was possible in this study as the animals were kept in groups of the same sex. In this case, it would have been helpful not to pool plasma for the LH measurements from both males and females, but due to the small amount of plasma and the small number of animals surviving until the sampling this was not possible.

However, these results indicate, that the effects of BPA on the sex steroids are not mediated only at the end-organ level. If the cause of the observed increase in the plasma LH value of the field voles had been decreased negative feedback of testosterone, a decrease in the circulating testosterone levels should have been observed. As the levels of both these hormones, however, increased in the field vole, it is possible that the effects of BPA were exerted at the level of LH. *In vitro*, in the presence of serum the affinity of BPA to the estrogen receptor is quite high (Nagel et al., 1997). It can be speculated that BPA could block the sex steroid receptors in the hypothalamus causing an increase in the circulating LH levels and thus stimulating sex steroid synthesis. Yet this remains highly speculative. As the estradiol and LH measurements of the voles had to be carried out from pooled plasma, these results need confirmation in future studies. Also the relatively small number of animals could have affected the outcome emphasizing the need to reconfirm the results in chronic, preferably peroral exposure studies.

The plasma cortisol levels of both species were mostly unaffected. The rationale behind the cortisol measurements was to investigate if the possible effects caused by BPA could be due to an increase in the stress level of the exposed animals. As the cortisol levels, especially in female polecats at 50 mg BPA kg⁻¹ d⁻¹, in fact decreased, the other observed effects were probably not due to stress.

The leptin levels of the polecats were not affected by BPA treatment. In the field voles,

plasma leptin and ghrelin levels were measured from pooled plasma (II) causing difficulties in the interpretation of the results. Ghrelin is an orexigenic hormone that increases food intake and increases obesity (Tschöp et al., 2000) – effects opposite to those of leptin (Pellemounter et al., 1995). Plasma ghrelin levels decrease in human obesity (Tschöp et al., 2001), while leptin levels increase (Maffei et al., 1995). In this study, the higher ghrelin values seen at high BPA doses were not reflected by any change in the BM of the animals and thus seem to be independent of BM. The small number of surviving experimental animals and the pooling of plasma from animals of both sexes makes the ghrelin results preliminary, and they will have to be examined individually in future studies.

6.2.3 Effects on biotransformation enzymes

In the polecats exposed to BPA, EROD activity was not altered. This conforms to previous results on the rat with no clear effect of BPA on the EROD activity after a 4-day exposure (Hanioka et al., 1998). Presumably the only mild to moderate toxicity (Alexander et al., 1988) of BPA did not require phase I activation. At the same time, the females had significantly higher EROD activities than the males. This fits to previous results, as in the rat it has been observed, that BPA suppresses especially the male-specific P450 isoforms (Hanioka et al., 1998), which could partly explain the higher EROD activities of female polecats.

The phase II reactions were, on the other hand, induced in the BPA-treated female polecats (I), indicating increased activity in the conjugation processes binding BPA to both glutathione and glucuronic acid (Armstrong, 1987). In evolutionary sense, the more pronounced phase II activation of females is reasonable in order to prevent teratogenic effects on the offspring. On the whole, however, the differences in the biotransformation enzyme activities between the BPA exposed groups and the control groups were relatively small compared to the hundred or thousand-fold elevations of these enzyme activities seen with many other xenobiotics (James, 1987). Thus this

nonexistent or very slight induction can be another sign of only moderately toxic effects of BPA (Alexander et al., 1988).

The effects of BPA on the biotransformation enzymes of the field vole (II) were quite different from this. BPA suppressed the liver EROD activity at all BPA doses in both sexes, and the liver GST activity was suppressed at 250 mg kg⁻¹ d⁻¹. This could be a nonspecific toxic effect of BPA on the voles' liver and biotransformation apparatus. The sc administration route makes the comparison to the polecat impossible, but in a similar sc study of Hanioka et al. (1998), a nonsignificant increase in the EROD activity was seen. Thus the effects of BPA on the biotransformation of mammalian species are different indicating to the need of using various species in risk assessment.

6.2.4 Summary of the effects of BPA

In the polecat and in the field vole the effects of BPA on the biotransformation enzymes were the opposite – slight induction in the polecat and suppression in the field vole. This difference can be due to the different administration route. Changes in the biotransformation enzyme activities were mostly less than 20% even at high BPA doses – a modest effect compared to more toxic xenobiotics conforming to the established model of BPA being only mildly to moderately toxic (Alexander et al., 1988). The increased mortality of the field voles was probably due to the sc administration route. Yet the higher mortality compared to previous studies indicates to the field vole being more susceptible to the toxic effects of BPA than e.g. the rat (Hanioka et al., 1998).

An increasing trend in plasma testosterone concentrations was encountered in both species due to BPA. In the field voles this was complicated by the contradictory and simultaneous increase in the pooled plasma LH value. Yet there is possibility that the effects of BPA could be exerted at the hypothalamic level. Generally, the effects of BPA on the endocrine parameters were not pronounced. BPA did not seem to be estrogenic *in vivo*, and all the effects could not be explained by estrogenicity only.

In the polecats with minor effects on many endocrine parameters and enzymes the discriminant analysis indicated to a deviation from the normal physiology between 10 and 50 mg kg⁻¹ d⁻¹. This is below the oral reference dose considered to be without deleterious effects during a human lifetime (U.S. EPA, 1987).

6.3 The effects of PS

6.3.1 General parameters

In the polecats PS exposure (III) did not have any effect on the BM, BMI, length, absolute or relative liver weight or testicular weight of the animals. In the field voles (IV), however, an increase in food intake was observed at 50 mg PS kg⁻¹ d⁻¹. This effect is probably not only due to the inhibition of cholesterol absorption in the intestine (Mattson et al., 1982). PS do not inhibit other lipids from being absorbed – a phenomenon, which could lead to increased appetite in order to meet the demand for energy. Furthermore, no differences were observed in the BM of the study groups. This increased food intake with no change in BM could indicate increased metabolic output – a hypothesis which has some support from the slightly elevated T₄ levels of the PS treated voles. Also a decrease in the relative liver weight could be seen at all PS doses. At the same time, the relative kidney weight decreased at 50 mg PS kg⁻¹ d⁻¹. These effects of PS have not been previously reported. They do not seem to be due to e.g. changes in the liver or kidney glycogen content as the glycogen concentration of these organs was not affected by PS. Thus their explanation requires further studies.

6.3.2 Endocrine effects

In the polecats (III), PS increased the plasma estradiol concentrations of both sexes (Table 4). In addition, plasma testosterone concentrations correlated positively with PS dose. Although the LH levels were not affected, there is a possibility of the hypothalamus influencing the observed increase in estradiol concentrations, as in both male and female polecats estradiol

exerts a negative feedback on LH secretion (Carroll and Baum, 1989).

In the field voles, too (IV, Table 5), the individual plasma estradiol levels increased at $5 \text{ mg kg}^{-1} \text{ d}^{-1}$. At the same time, the plasma LH value measured from pooled plasma decreased. This could be due to negative feedback caused by the increasing circulating estradiol concentrations. These effects are different from previous observations on fish with decreased sex hormone levels due to PS (MacLachy and Van der Kraak, 1994). As the hypophyseal regulatory hormones of the polecats were not affected, these effects seem to be mediated at the end-organ level in the polecat. Generally the effects on the sex steroids of both polecats and voles were of a similar kind and could indicate to increased estradiol (and testosterone) synthesis in gonads from PS precursors (Aringer et al., 1979; Moghadasian, 2000). In mice and rats PS have been detected in steroid-producing glands (Moghadasian, 2000; Sanders et al., 2000), where they can be used by the organism as precursors of sex steroids.

Photoperiod could also have a significant effect on the circulating sex steroid levels of these species (Jallageas et al., 1994). This seems, however, unlikely. In the case of the polecats, the study period was in November-December, before the darkest part of the year. It was the season of a shortening photoperiod with less than 8 hours of daylight, which would be required for testicular activation (Jallageas et al., 1994). The voles, on the other hand, were housed in a constant photoperiod. Thus the observed effects do not simply reflect the seasonal endocrine status of the species.

In the thyroid axis of the polecats, the T_4 concentrations of males increased at 5 or $50 \text{ mg PS kg}^{-1} \text{ d}^{-1}$. As the T_3/T_4 ratio increased at the same time, this indicates to both increased synthesis of T_4 and increased deiodination of T_4 to T_3 . In the field voles, again, the picture was slightly different. The slight increase in plasma T_4 concentrations remained nonsignificant, but the TSH level measured from pooled plasma dropped to undetectable levels at the PS exposed groups. Like with the plasma estradiol levels, this could be due to increased negative feedback caused by T_4 on the hypophysis.

The effects of PS on the thyroid axis have not been studied previously. The results indicate to activation of the thyroid due to PS – a phenomenon that should be more thoroughly investigated in chronic exposure studies. In the case of the field voles, the small number of animals ($n = 31$) and the pooled TSH plasma samples could have masked the effects.

Ghrelin is the newly-discovered peptide (Kojima et al., 1999) increasing food intake and BM of rodents (Tschöp et al., 2000). The plasma ghrelin concentrations of the polecats (III) decreased at $50 \text{ mg PS kg}^{-1} \text{ d}^{-1}$. This decrease occurred independent of leptin or body adiposity. Thus the effects of PS on plasma ghrelin levels seem to be mediated by other mechanisms, perhaps by a direct effect of PS on ghrelin secretion. The plasma leptin concentrations of the polecats were not affected by PS, but the results conformed to the established model of leptin being an indicator of body adiposity with a positive correlation between the polecats' leptin levels and BMIs (Maffei et al., 1995; Ma et al., 1996).

In the field voles (IV), the plasma leptin and ghrelin values measured from pooled plasma changed to opposite directions at $5 \text{ mg PS kg}^{-1} \text{ d}^{-1}$. A similar effect was observed in study II in voles exposed to a high BPA dose. At $5 \text{ mg PS kg}^{-1} \text{ d}^{-1}$, the leptin value increased and, at the same dose, the ghrelin value decreased. However, in this parameter, too, the effects of PS on the field vole were biphasic with an increase or a decrease in the circulating hormone concentration at $5 \text{ mg kg}^{-1} \text{ d}^{-1}$ and a return to the level of control animals at a higher dose. The actual differences in circulating leptin and ghrelin concentrations in pooled plasma were quite small, especially in the case of ghrelin. The theory of leptin and ghrelin being antagonistic of each other (Shintani et al., 2001), however, suggests that these preliminary result could indicate to an actual effects of PS and BPA on hormonal weight regulation. As these hormone levels were determined from pooled plasma, these results, too, are only preliminary and need to be reconfirmed in the future.

6.3.3 Enzymatic effects

The biotransformation enzyme activities measured in these studies showed no effect of PS on the field vole, and in the polecat the effects were minor, too (III-IV). Female polecats had higher liver GST activities than the males, a phenomenon which could also be observed with BPA exposure (I). The small observed differences in the voles can, again, be caused by chance fluctuation in the small number of experimental animals.

Alltogether, however, PS did not seem to be toxic or recognized as a foreign substance. Thus the biotransformation apparatus was not activated. Previously PS have either stimulated or suppressed EROD activity in fish (Mattsson et al., 2001b). In the rainbow trout these effects have been biphasic – induction at low or high doses but suppression at intermediate doses. This phenomenon seems to be similar to the many biphasic effects observed in the field vole (IV).

The carbohydrate metabolism of the polecat changed significantly with PS exposure (III). The liver glycogen content increased at 50 mg PS kg⁻¹ d⁻¹. This could indicate a direct effect of PS on liver intermediary metabolism. Furthermore, in female polecats, the kidney G6Pase activity increased due to PS treatment. G6Pase correlates with gluconeogenic activity (Harris, 1986) and provides energy mainly for the kidney itself. Yet, at the same time, also the kidney glycogen phosphorylase activity increased. As gluconeogenesis is needed at times of energy shortage, this could indicate decreased energy availability to the kidneys due to PS exposure requiring increased release of glucose from glycogen and increased carbohydrate turnover.

In the field vole the effects were, again, biphasic (IV). Both the liver G6Pase activity and the glycogen phosphorylase activity were the highest at 5 mg kg⁻¹ d⁻¹. At the same time, the relative liver weights of the voles decreased at both PS doses. These data indicate to enzyme induction and increased carbohydrate turnover in the liver at 5 mg kg⁻¹ d⁻¹ with a return to the level of the control animals at a higher dose. In the field voles like in the polecats this suggests direct effects of PS on intermediary metabolism.

6.3.4 Effects of PS on serum lipids of the polecat

The principal use of PS in medicine is to lower elevated serum total and LDL cholesterol levels in order to reduce the risk of cardiovascular disease. This effect of PS on serum cholesterol levels has been thoroughly documented in humans (Vanhanen et al., 1993; Miettinen et al., 1995; Gylling et al., 1997; Weststrate and Meijer, 1998; Hallikainen et al., 2000; Jones et al., 2000; Vissers et al., 2000; Neil et al., 2001).

In contrast to humans, the serum LDL cholesterol levels increased in the polecats due to PS. At the same time, the HDL/cholesterol ratio decreased. The serum cholesterol and triglyceride levels of the polecats were quite high compared to healthy humans. Autumnal fattening is, however, physiological to the polecat, and the study period coincided with this seasonal weight gain (Korhonen and Harri, 1986). This seasonal increase in food intake can partly explain the relatively high serum lipid levels.

Furthermore, the HDL cholesterol concentrations of the polecats were very high compared to humans, a phenomenon described also previously (Cryer and Sawyerr, 1978). In fact, in ferrets (the semi-domesticated subspecies) HDL is the lipoprotein fraction in which most of the serum cholesterol is present. This is different from the human cholesterol transport system but similar to the dog (Solyom et al., 1971). Thus the HDLs of humans and polecats have approximately the same density but a different physiological function, the significance of which remains unknown.

Unfortunately the results of this study do not offer an explanation, why reduced cholesterol uptake in the intestine would increase the LDL cholesterol levels in polecat serum. As the lipoprotein metabolism of carnivores differs drastically from humans, this effect cannot be described as deleterious as it would be in medicine. Of course, it can be hypothesized that the increase in LDL levels can only be due to increased cholesterol synthesis in the liver, a phenomenon observed also in hamsters (Ntanios and Jones, 1999), mice (Moghadasian et al., 2001) and humans (Vanhanen et al., 1993) due

to PS intake.

The activity of liver lipase esterase decreased in the polecats at 5 or 50 mg PS kg⁻¹ d⁻¹. This in concert with previous observations on the mouse with a reduction in hepatic lipase activity due to β -sitosterol (Moghadasian, 2000). It is still unknown, whether these effects of PS on the serum lipoproteins and on hepatic lipase esterase are direct or caused by decreased cholesterol uptake in the intestine. The decreased lipase esterase activity could, of course, mean that less lipids are used for metabolic energy, which could partly explain the increase in liver glycogen content as more of the energy had to come from carbohydrates.

6.3.5 Summary of the effects of PS

The effects of PS have not previously been studied in many wild-type mammals and not at all in the polecat or the field vole. Thus it is understandable that the effects of PS on the physiology of the polecat and the field vole were previously unreported. In the polecats, the effects could generally be observed at a fairly high PS dose, but in the field voles the effects were most obvious at 5 mg PS kg⁻¹ d⁻¹. This was an unexpected observation but slightly similar to previous results in fish (Mattsson et al., 2001b).

It is possible, that the small number of individual voles (n = 31) and the large interindividual variation in the results, especially in the sex steroid measurements, could have caused the effects instead of actual physiological changes. On the other hand, all the statistically significant effects, except the increase in food intake, were observed in the same dose group, 5 mg PS kg⁻¹ d⁻¹. This indicates that a physiological change could indeed have taken place with a return to the metabolic state of the control animals at a higher dose. In the case of the field vole, it is possible that at higher PS doses other mechanisms besides the liver biotransformation counteract the effects of PS successfully. In this regard, the field vole as a herbivore indeed seems to be better adapted to PS as a part of its diet than the carnivorous polecat is.

6.4 General implications

6.4.1 Comparison between the effects of BPA and PS

The results of these studies indicate that all the effects of BPA or PS are not due to their estrogenicity. Not only were the sex steroid concentrations or the levels of their hypophyseal regulatory hormones affected by one or both of these compounds. There were also previously unreported changes in intermediary metabolism

In general, the effects of PS were stronger on the endocrine system of these species than the effects of BPA were. BPA, on the other hand, caused similar but not as pronounced effects on the concentrations of various hormones. A common nominator was an increase in the circulating testosterone levels and a slight increase in the circulating T₄ levels of male polecats.

In the case of PS, it is possible that the observed increases in the circulating sex steroid levels could be caused by increased sex steroid synthesis from PS precursors. With BPA this is impossible as the molecule cannot be used in hormone synthesis (Fig. 1). Thus, at least in the case of BPA, there are probably other mechanisms behind these effects on the sex steroids.

Table 6. Comparison between the effects of BPA and PS

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- The effects of PS on endocrine systems are more pronounced than the effects of BPA
 - The effects of BPA on biotransformation are stronger than the effects of PS
 - Both compounds cause an increasing trend in plasma testosterone concentrations
 - Estradiol levels increase except in the BPA treated polecats
 - The effects appear at relatively low doses: below the oral reference dose in the case of BPA, and at a therapeutic dose used to lower serum total cholesterol in the case of PS
 - The effects of PS on the polecat are mostly linear, but the effects of PS on the field vole mostly biphasic
-

In the biotransformation enzymes the effects of BPA were more pronounced. In the polecats there was some induction of phase II reactions,

especially in the females. In the field voles the result was a slight suppression, possibly due to nonspecific toxic effects of BPA. It is ecologically sound that a top predator – the polecat – should have a more efficient biotransformation apparatus as it is more at risk of bioaccumulation than the small field vole at a lower trophic level.

Furthermore, the results of this study indicate that PS do not induce the xenobiotic transformation reactions catalyzed by the CYP1A enzyme family. PS do not seem to be recognized as foreign compounds unlike chemical xenoestrogens. Thus they can affect the organism without any interference from the biotransformation apparatus.

6.4.2 Biomonitoring and risk-assessment

These studies yielded no easily accessible biomarkers for monitoring the effects of BPA or PS in nature. Instead, there were many different effects, some of which may be due to methodological limitations and none of which is specific to either BPA or PS. In addition, some of the effects were puzzling or even contradictory (e.g. the increase in both plasma testosterone levels and LH value in study II).

In the case of the BPA exposed polecats, the effects were in general minor and only the discriminant analysis suggested that the overall physiology of the exposed animals had changed due to BPA. The effects of endocrine disruption observed in aquatic ecosystems is better defined with intersexuality, deformations and gross changes in the animals' sex steroid levels (see e.g. Noaksson et al., 2001). Yet the exposure was only subacute, and chronic exposure studies are definitely needed to establish, if these effects would prove to be hazardous in the long run.

Extrapolation between species is difficult and great prudence should be employed when considering the use of these results in human risk assessment. The effects of BPA and PS were quite different in the two species studied in this thesis. It is conceivable that they would find yet another form in humans. The well documented effect of PS on serum cholesterol levels is an example of a phenomenon that is

just as opposite in the polecat and in *Homo sapiens* as their lipoprotein system are (Cryer and Sawyerr, 1978). In fact, none of the effects observed in the polecat or the field vole were *per se* deleterious, harmful or, on the other hand, beneficial.

Yet the many effects in various physiological parameters should not be taken too lightly, either. The effects of peroral BPA classified the exposed polecats apart from their controls at a level below the oral reference dose – 50 mg BPA kg⁻¹ d⁻¹ – supposed to be without deleterious effects if consumed daily during a human lifetime. PS caused multiple slight effects at doses considered to be therapeutic in PS containing spreads. In the field voles, the toxic effects of BPA were quite pronounced. There are also possible interactions and synergistic effects between other plant sterols that the general public consumes in diverse plant material.

Do bisphenol A or phytosterols classify as endocrine disruptors or "exogenous substances that cause adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function" in the polecat and the field vole? In the case of the field vole and sc BPA the answer should probably be "yes" due to increased mortality. In the other studies the answer is "maybe" as the word "adverse" needs to be looked into in future studies.

7 CONCLUSIONS

These conclusions are drawn from the studies I-IV.

1. Both BPA and PS increase the testosterone concentrations of polecats and field voles. PS also elevate the plasma estradiol concentrations.
2. BPA and PS slightly increase the circulating T₄ levels of male polecats, while PS cause a decrease in plasma ghrelin concentrations. Most of the effects of PS on the field vole are biphasic.
3. The effects of PS on the biotransformation enzymes are minor. Also the effects of BPA are relatively small. BPA increases the activity of

phase II reactions in female polecats, but suppresses biotransformation in the field vole.

4. PS increase the liver glycogen content and the rate of carbohydrate turnover. They also elevate the serum LDL cholesterol levels of polecats and decrease their hepatic lipase esterase activity.

5. No practical biomarkers for environmental monitoring or risk assessment could be found. The effects of the substances targeted on various physiological parameters, but were unspecific.

6. The effects of BPA and PS on these species were previously unreported, differed from each other and from previous observations on more traditional experimental animals. The use of wild-type mammals in toxicity tests may prove to be valuable.

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