

**Endocrine potential of  
Genistein, Resveratrol and 8-Prenylnaringenin  
in gonadal and thyroid axes and related organs**

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**Das endokrine Potential von  
Genistein, Resveratrol und 8-Prenylnaringenin in der Gonaden-  
und Schilddrüsenachse und assoziierten Organen**

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## Summary

Phytoestrogens are increasingly consumed in high doses as herbal preparations and nutritional supplements. So far they are, in contrast to pharmaceuticals, not subjected to stringent regulatory safety requirements concerning their potential endocrine activity under long term use by unenlightened customers.

Three phytoestrogens belonging to different structural classes were investigated. The isoflavonoid genistein - contained in soy products - is marketed as substitute for classical hormone replacement therapy or as cancer preventive. The stilbene compound and red wine constituent resveratrol is mainly promoted with claims for cardiovascular protection, and the flavanone 8-prenylnaringenin, component of hops, was identified as the most potent phytoestrogen known so far and is an active constituent of preparations for bust enhancement.

A series of experiments was designed to investigate the influence of the test compounds on the regulation of the gonadal and thyroid axes in vitro and in vivo. Pituitary cell culture and the female ovariectomized rat, a model for postmenopausal conditions, were used.

The substances were tested for

- effects on LH and TSH release under in vitro conditions,
- response of the two axes in vivo after a single high dose challenge and
- permanent or adaptive changes of the hormonal regulation respectively related organs like the reproductive tract, metabolic activity of the liver and bone condition after three months treatment with two doses to detect potential organ selectivity.

Genistein showed variable responses in the pituitary and the thyroid gland. In vitro it did not show any influence on the pituitary hormones LH and TSH. In vivo it suppressed LH secretion and increased TSH release after administration of a single high dose, but had no effect on LH levels and caused a lowered level of free T3 in the long term.

Estrogenic effects reported on bone density could be verified, but not the effects concerning reduction of cholesterol and LDL levels. Alterations detected in the reproductive organs were not purely of estrogenic origin.

Resveratrol did not cause significant estrogenic effects in the gonadal axis or in any of the related organs in the postmenopause animal model. But it revealed effects on the thyroid hormones T3, T4 and calcitonin, though the thyroid axis regulation adapted to an euthyroid status in the long term.

The strong estrogenic potency of 8-prenylnaringenin was substantiated in persisting LH suppression, positive effects on lipid metabolism and improved bone density.

The respective rise of TSH in cell culture experiments and after single dose application was normalized in the long term. On the other hand reproductive organs displayed features that cannot be explained solely by estrogenic effects.

Depending on experimental model, dosage and exposition time the test substances displayed a complex picture of their influence on various variables.

These findings show that it may be misleading to use the sum of single observed parameters for the deduction of a general mechanism of action.

Potential use in therapy and prevention bears the risks of undesired side effects.

Caution is advisable regarding the long term consumption of genistein, resveratrol or 8-prenylnaringenin as nutritional supplements or herbal preparations. Before admitting a free access of customers to such products containing high concentrations of these compounds thorough safety assessment of the risk-benefit ratio is highly recommended.

## Zusammenfassung

Phytoestrogene werden in zunehmendem Maß in hohen Dosen von einer weiten Bevölkerungsschicht als pflanzliche Zubereitungen oder Nahrungsergänzungsmittel konsumiert. Im Gegensatz zu Arzneimitteln sind diese Produkte praktisch keiner Regulierung unterworfen, die einen Nachweis der Sicherheit ihrer möglichen endokrinen Aktivität während einer langfristigen Anwendung durch unaufgeklärte Kunden erfordert.

In der vorliegenden Arbeit wurden drei Phytoestrogene untersucht, die zu verschiedenen strukturellen Klassen gehören. Das Isoflavonoid Genistein ist in Sojaprodukten enthalten und wird als Alternative zur klassischen Hormonersatztherapie oder zur Krebsvorsorge vermarktet. Das Stilbenderivat trans-Resveratrol, natürlicherweise in Rotwein vorhanden, wird hauptsächlich unter Anspruch auf antioxidative Wirkung zum Schutz des Herz-Kreislauf-Systems angeboten. Das Flavanon 8-Prenylnaringenin, ein Inhaltsstoff von Hopfen, wurde als das stärkste bisher bekannte Phytoestrogen identifiziert und ist ein Bestandteil von Präparaten, die zur Brustvergrößerung offeriert werden.

Es wurde eine Folge aufeinander aufbauender Experimente entworfen, die es erlauben sollten, den Einfluß der Testsubstanzen auf die hormonelle Regulation der Gonaden- und Schilddrüsenachse *in vitro* und *in vivo* zu untersuchen. Zum Versuch dienten eine Hypophysenzellkultur und die ovariectomierte Ratte als Modell für postmenopausale Bedingungen. Die Substanzen wurden dosisabhängig getestet auf ihre

- Effekte auf LH- und TSH-Freisetzung *in vitro*,
- Antwort der beiden Achsen *in vivo* auf eine einmalige hochdosierte Belastung und
- Reaktion - anhaltende Veränderung oder Anpassung - der hormonellen Regelmechanismen, des Reproduktionstraktes und assoziierter Organe wie der Leber mit ihrer metabolischen Aktivität und der Zustand des Knochens nach drei Monaten Behandlungszeit mit zwei Dosen zur Entdeckung möglicher Organselektivität.

Genistein zeigte unterschiedliche Wirkungen in der Hypophyse und Schilddrüse. *In vitro* fand sich kein Einfluß auf die Hypophysenhormone LH und TSH. *In vivo* wurde nach einer hohen Einmaldosis die Freisetzung von LH unterdrückt, diejenige von TSH erhöht, aber im Langzeitversuch hatte die Substanz keine Wirkung auf den LH-Spiegel und verursachte einen erniedrigten Spiegel an freiem T3. Die Berichte von estrogenen Effekten auf die Knochendichte konnten bestätigt werden, nicht aber die Senkung der Plasmaspiegel von Cholesterin und LDL. Veränderungen, die im Reproduktionstrakt beobachtet wurden, waren nicht ausschliesslich estrogenen Ursprungs.



Resveratrol verursachte keine signifikanten estrogenen Effekte in der Gonadenachse oder einem der angegliederten Organe in dem verwendeten Postmenopause-Tiermodell.

Es entwickelten sich jedoch deutliche Wirkungen auf das Sekretionsmuster der Schilddrüsenhormone T3, T4 und Calcitonin, wobei die Schilddrüsenachse im Langzeitexperiment einen euthyroiden Status einstellte.

Die hohe estrogenen Potenz von 8-Prenylnaringenin wurde durch anhaltende LH-Suppression, positive Wirkungen auf den Fettstoffwechsel und verbesserte Knochendichte nachgewiesen. Der jeweilige Anstieg von TSH in der Zellkultur und nach Einmalgabe normalisierte sich nach der Langzeitapplikation. Auf der anderen Seite wiesen die Reproduktionsorgane histologische Merkmale auf, die nicht nur durch estrogenen Effekte erklärt werden können.

Abhängig von experimentellem Modell, Dosis und Expositionsdauer bietet sich ein komplexes Bild der Einflüsse der Testsubstanzen auf verschiedene Variablen. Die Befunde zeigen, dass die Summe einzelner beobachteter Parameter nicht auf einen einzigen, generellen Wirkungsmechanismus schließen lässt.

Die Möglichkeit zur Anwendung in Therapie oder Prävention birgt auch die Risiken unerwünschter Nebenwirkungen. Vorsicht und Zurückhaltung sind daher angeraten bei langfristiger, unkontrollierter Einnahme von Genistein, Resveratrol oder 8-Prenylnaringenin in Form von Nahrungsergänzungsmitteln oder pflanzlichen Zubereitungen. Ehe ein unkontrollierter Zugang des Kunden zu solchen Produkten erlaubt wird, die diese Substanzen in hohen Konzentrationen enthalten, ist eine sorgfältige behördliche Abschätzung des Nutzen-Risiko-Verhältnisses dringend empfohlen.

# 1 Introduction

## 1.1 Background information

When research identifies positive effects in food components, for example cancer preventive, cardio-protective or other beneficial properties, there is a new trend to isolate the component (or use the whole plant) and offer it to the customer in a high concentration in a tablet. This approach is called nutritional supplement or herbal remedy and they are promoted as “natural compounds”, since the products are originally derived from normal food sources. On the other hand the dosage has to be considered as very “unnatural”, i.e. the amount of these compounds in one tablet, often exceeds the amount ingested by normal and “natural” consumption. In most people’s perception any product derived from nature is harmless and an increase of concentration enhances the positive effects. Uncritical use bears risks, and so far there is no valid law in Germany or the EU to protect consumers’ health in this grey zone between food and drug regulations. Many nutritional supplements and herbal remedies should be classified as drugs, but without risk versus benefit evaluation authorities cannot take action. On the other hand the internet offers an enormous variety of products from dubious sources, that are not subjected to any quality control but which are accessible to anyone. Thus national regulations may be of limited value.

The substances investigated in this work, genistein (GEN), *trans*-resveratrol (RES) and 8-prenylnaringenin (8PN) are constituents of nutrition sources, but are also offered in high concentrations. All three test compounds belong to the group of the so called phytoestrogens, and are also referred to as “naturally occurring non-steroidal estrogenic substances” (NONEs). GEN, RES and 8PN are known to bind to two subtypes of the estrogen receptor (ER), ER $\alpha$  and ER $\beta$ , whose existence is well established (Kuiper *et al.* 1996). But they have different affinities and develop estrogenic and/or antiestrogenic effects in different organs.

### Genistein

GEN is an isoflavone predominantly found in soy products. The traditional vegetarian Asian diet provides lifelong exposure to fairly high levels of soy isoflavones. Several studies suggest that there is a reduced prevalence of breast cancer in those Asian populations that cover their protein needs through soy products (Stephens 1997; Adlercreutz 1998). This hypothesis is substantiated by the observation that after exposure to typical western diet Asian women lose this protection in the second generation (Ziegler *et al.* 1993).

In 2002 the estrogen/progestin (conjugated estrogens plus medroxyprogesterone) arm of the Women's Health Initiative (WHI) Study in the U.S. was prematurely stopped because adverse effects were outweighing beneficial effects (Rossouw *et al.* 2002). Negative reports from the "Million Women Study" in England 2003 added to the trend against classical hormone replacement therapy (HRT) (Beral 2003). Estradiol and its derivatives are most effective in suppressing severe climacteric complaints, also slowing down or even preventing osteoporosis. Increased risk of mammary cancer, stroke and thromboembolic incidents is the drawback of long term treatment. If progestins are not included in the substitution, endometrial cancer risk rises as well (Nelson *et al.* 2002). Many women stopped their HRT and seek alternatives now, preferably "natural" plant derived substances, as opposed to any estrogen presumed unnatural. Thus, soy nutritional supplements containing GEN are increasingly offered to diminish climacteric and postmenopausal complaints. Yet none has acquired approval as a drug, which seems logical considering the contradicting scientific results (Vincent *et al.* 2000). ER $\alpha$  and ER $\beta$  binding studies showed a stronger activity of GEN at the ER $\beta$  (Kuiper *et al.* 1997), thus supporting the idea of a potential selective estrogen receptor modulator (SERM) acting mainly in bone and brain (Patisaul *et al.* 2002; Setchell *et al.* 2003). But besides the positive influence on bone density GEN has uterotrophic effects (Diel *et al.* 2001), stimulates proliferation of human mammary carcinoma cells (in vitro and in transplanted animals) and it reduces hot flushes only slightly, if at all (Ju *et al.* 2001; Maggiolini *et al.* 2001; Van Patten *et al.* 2002). Developmental studies also suggest that the prevention of mammary cancer depends on soy consumption throughout life and cannot be obtained in later years, when GEN might even develop adverse influence (Fritz *et al.* 1998; Lamartiniere *et al.* 2002; Allred *et al.* 2004). Therefore its benefits as a nutritional supplement remain questionable and require further investigation.

### **Resveratrol**

RES is a polyphenol of the stilbene class that is found in more than 70 common plant species (Turner *et al.* 1999). Main dietary sources for humans are grapes (especially skin), peanuts and their products.

The list of unapproved health claims for the numerous nutritional supplements includes protection from free-radical damage of blood vessels against cardiovascular disease and cancer, inhibition of arthritic inflammation and alleviation of menopausal symptoms (Haneke 2002). The „French paradox“, i.e. low rate of cardiovascular incidences in spite of high calory diet, is often assigned to the regular consumption of red wine and its RES content (Kopp 1998; Wu *et al.* 2001).

RES exerts mixed agonist/antagonist activities. It binds equally to both ER subtypes and depending on estrogen response element (ERE)-sequence inhibits ER $\alpha$  but not ER $\beta$  transcriptional activity (Bowers *et al.* 2000), whereas in presence of 17 $\beta$ -estradiol it functions as an antiestrogen (Bhat KP *et al.* 2001). Anticarcinogenicity was shown in human mammary carcinoma and prostate cancer cell lines where growth was inhibited and in rat and mouse cancer induction models (Haneke 2002). Antioxidant activity in LDL metabolism, inhibition of platelet aggregation and neuroprotective effects have been reported in vitro (Bhat KPL *et al.* 2001; Hao *et al.* 2004). To reproduce these findings in vivo proved to be difficult. No uterine estrogenic response could be detected in ovariectomized (ovx) rats (Freyberger *et al.* 2001), but disruption of the gonadal axis in intact rats was reported (Henry *et al.* 2002).

No clinical trials with RES were conducted so far, and thorough research for the pharmacological and clinical effects of high dose supplementation effects is still necessary.

### **8-Prenylnaringenin**

The flavanone 8PN was identified as potent phytoestrogen in a Thai crude drug (Kitaoka *et al.* 1998) and as the major estrogenic compound in female hop cones (Milligan *et al.* 1999). Therefore considerable amounts are found in beer, though the content varies depending on the hop strain used for brewing. In the last years hop increasingly appears as a component of herbal preparations for “bust enhancement”, that are mainly available via the internet (Fugh-Berman 2003), and 8PN proved to be their main estrogenic ingredient (Coldham *et al.* 2001).

Customers for these products will be most prone to unlimited use. Medical research with this substance started only in recent years, and fairly little information is available in comparison to GEN and RES. But it is clear that 8PN binds to both ER $\alpha$  and ER $\beta$  and develops the strongest ER $\alpha$  activity detected for a phytoestrogen so far (Bovee *et al.* 2004). In vitro tests showed 8PN to be a potent and selective inhibitor of human cytochrome P-450 mediated reactions that activate carcinogens (Henderson *et al.* 2000). Lack of androgenic and progestogenic (Milligan *et al.* 2000), but antiandrogenic activity was demonstrated (Zierau *et al.* 2003). 8PN also inhibits angiogenesis in vitro and in vivo (Pepper *et al.* 2004). In studies for its estrogenic activity in vivo it caused uterotrophic response and positive influence on bone density (Miyamoto *et al.* 1998; Diel *et al.* 2004), though not in lower concentrations (Coldham *et al.* 2001).

Due to the estrogenic potency of 8PN, safety concerns arise for unrestricted long-term use as a herbal preparation without drug status, and the need of further knowledge about positive and negative effects in vivo is obvious.

## 1.2 Purpose of the study

In the recent years campaigns to screen substances for endocrine disruption are initiated and funded by the EU commission research section, by the National Institute of Environmental Health Sciences (National Toxicology Program) and Environmental Protection Agency in the U.S. (Endocrine Disruptor Research Initiative), various other national programs and international authorities like the OECD, WHO and UN. The established priority lists for further evaluation of their role in endocrine disruption do not only include synthetic chemicals, but many phytoestrogens as well.

This study was performed within the framework of EURISKED (<http://www.eurisked.org/>) to assess the multi-organic risk of selected endocrine disruptors. The project is part the CREDO cluster (<http://www.credocluster.info/>). That represents over 60 groups of European research teams all investigating various aspects of endocrine disruption.

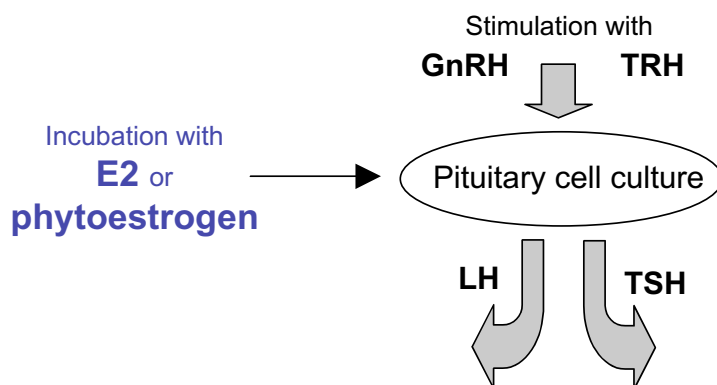
Substances known to exert estrogenic or antiestrogenic activity are usually investigated for their impact on the gonadal axis, which is very sensitive to disruption. For all three test compounds studies were already conducted covering that aspect. Due to efforts of authorities in the EU, the United States and the OECD testing protocols and guidelines were developed (Endocrine disruptors testing and assessment task force, EDTA). In the meantime there is consensus that looking for endocrine disruption must not be reduced to the hypothalamo-pituitary-gonadal (HPG)-axis, reproductive and related organs, but should also include other regulatory axes such as hypothalamo-pituitary-thyroid (HPT) and hypothalamo-pituitary-adrenals (HPA). Particularly the HPT-axis moved into focus, since thyroid hormones play a critical role in development and function of several organ systems like the nervous, cardiovascular, immune and reproductive system (DeVito *et al.* 1999; Doufas *et al.* 2000).

In vitro test systems provide only limited information on potential effects in vivo. Outcomes of in vivo study may vary depending on the duration of exposure, because the organism often adapts hormonal regulation mechanisms within a certain time frame. Nutritional supplements or herbal preparations may be used for extended periods, up to many years. To mimic such time spans in vivo is difficult, and only few studies exceed 60 days. All these aspects of endocrine potential of the test substances were covered by a series of three different experimental setups. The endocrine potential of GEN, RES and 8PN in the gonadal and thyroid axes was tested in vitro, as well as in vivo after a single dose and 3 months treatment. The long term treatment in vivo should supply information about changes in organs related to both axes under long lasting exposure. For the in vivo experiments animals were specifically bred under soyfree conditions to avoid any confounding influences of dietary estrogens (Degen *et al.* 2002; Lamartiniere 2002).

### 1.3 Cell culture

#### Experimental design

Pituitary cell culture was selected to investigate effects at the pituitary level of the HPG- and HPT-axes. Anterior pituitary cells were incubated with the three test compounds alone or in combination with the pure antiestrogen ICI 182,780 and stimulated by GnRH and TRH addition.



*Figure 1-1: In vitro assessment of direct pituitary action of estrogenic substances in the presence of releasing hormones. The secretion of gonadotropin and thyrotropin can be measured without confounding influences of feedback mechanisms.*

Preparation of the cells and incubation procedure followed previously published protocols investigating pituitary effects and regulation within the gonadal axis (Jarry *et al.* 1994; Ortmann *et al.* 1999).

#### Criteria for action in the HPG- and HPT-axes

LH and TSH content of the supernatants of the cell cultures were determined. Variation of LH and/or TSH concentration and/or antagonizing effect of ICI were considered as indicators for action at the pituitary level in the respective axis.

### 1.4 Single dose treatment

#### Experimental design

The ovariectomized rat is an established *in vivo* model which is highly sensitive for estrogenic activity in the HPG-axis. Four weeks after ovariectomy the rat maintains a serum level of constant high LH due to the lack of suppressing feedback by E2. A single high dose of the test substances was supposed to challenge the endocrine regulation of gonadal and thyroid axes.

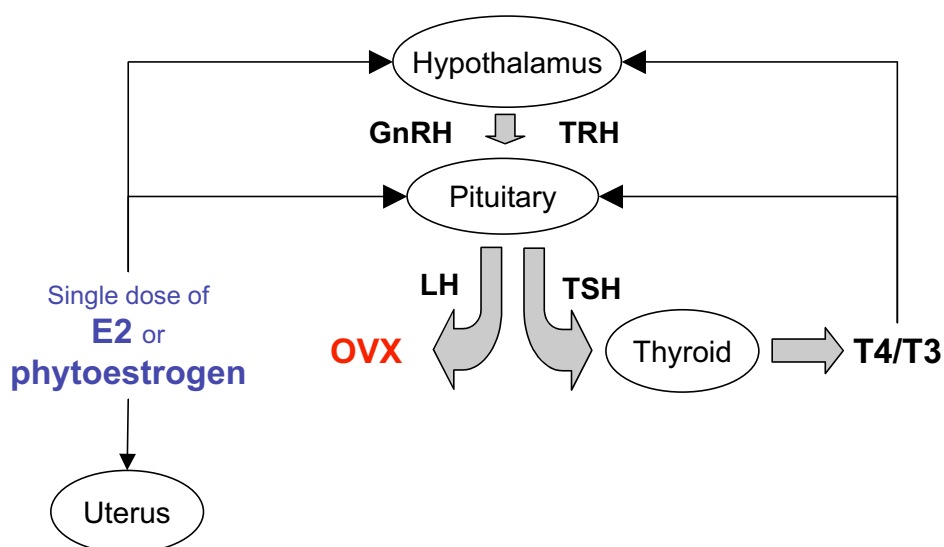


Figure 1-2: In vivo challenge of HPG- and HPT-axes by application of a single high dose of the test compounds to the animal. Uterus is the only target organ that might respond to an exposure time of a few hours.

The test substances were administered to the rats orally to imitate the usual route of administration including resorption restrictions, influence of intestinal flora and first pass metabolism. Thus, a 1-2h delay of systemic availability had to be calculated. To obtain information on alterations of LH and TSH serum levels, serial samples were drawn at several time points before and during the 6h after application.

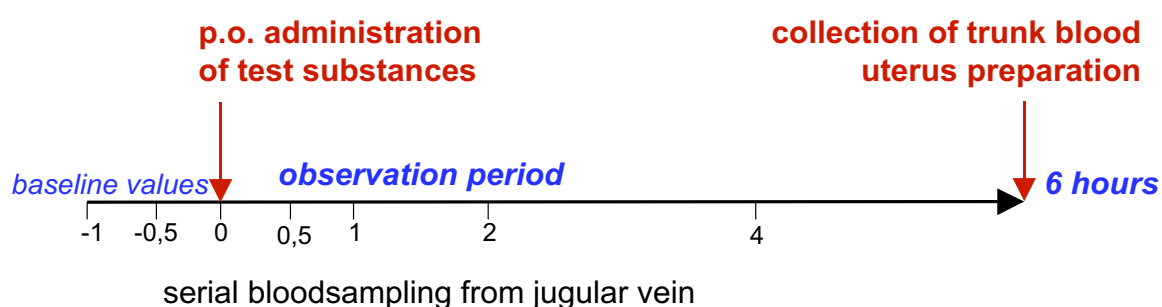


Figure 1-3: Time schedule of the single dose treatment.

GEN, RES and 8PN were applied in a high dose of 150mg/kg bw to achieve an effective challenge. E2 was chosen as positive control and applied with 15mg/kg bw to ensure sufficient absorption since its oral bioavailability is low (Schleicher *et al.* 1998).

### Criteria for action in the HPG- and HPT-axes

Increase in uterine weight and reduction of LH levels in serial samples and decapitation blood were regarded as “estrogenic activity” in the gonadal axis, while effects on TSH levels in serial samples/decapitation blood and on free and protein-bound (total) thyroid hormone concentrations in decapitation blood were considered as influence on the thyroid axis.

## 1.5 Long term treatment for three months

### Experimental design

To judge whether the endocrine regulation of gonadal and thyroid axes is altered or counterregulated during constant hormonal pressure long term treatment is necessary. Effects on reproductive organs, thyroid, liver and metabolic parameters are accessible. The ovx rat is also a good model for estrogenic activity in the bone as it develops osteoporosis induced by lack of E2 within a few weeks. Thus long term influence on bone condition can certainly be measured after three months treatment.

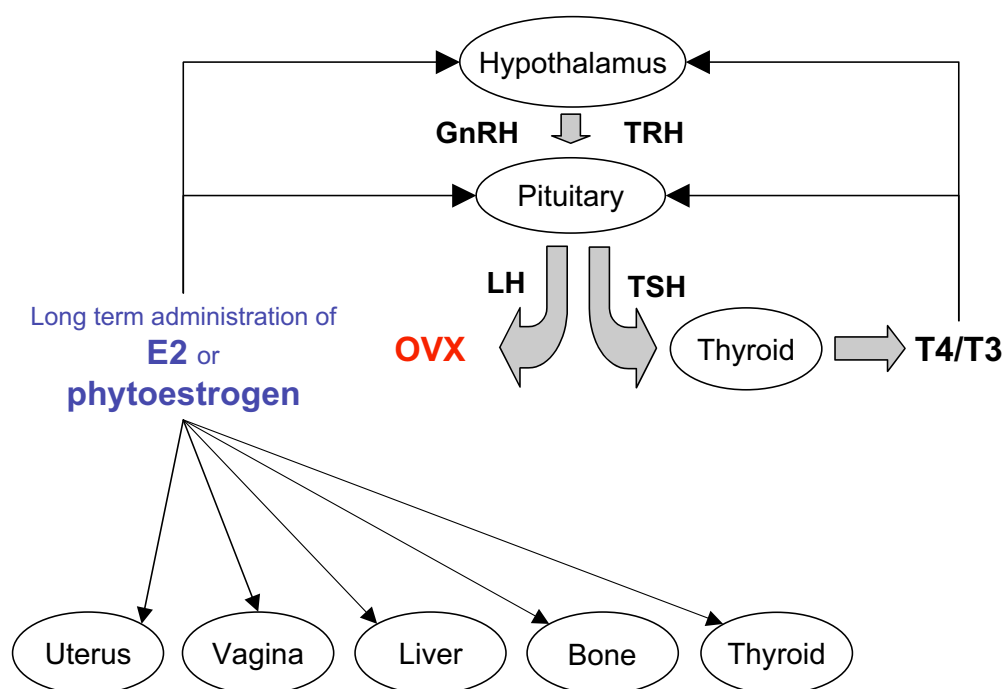


Figure 1-4: In vivo model to test endocrine potential in HPG- and HPT-axes and target organs that might undergo changes after extended exposure to the test compounds.



Like in the single dose treatment the test substances were given orally as nutritional supplements or herbal remedies are ingested by that route. The administration started with the day of ovariectomy (ovx). Bone mineral density at the metaphysis of the tibia was measured by quantitative computer-assisted tomography before and at the end of treatment. An age matched intact group was included for the main parameters.

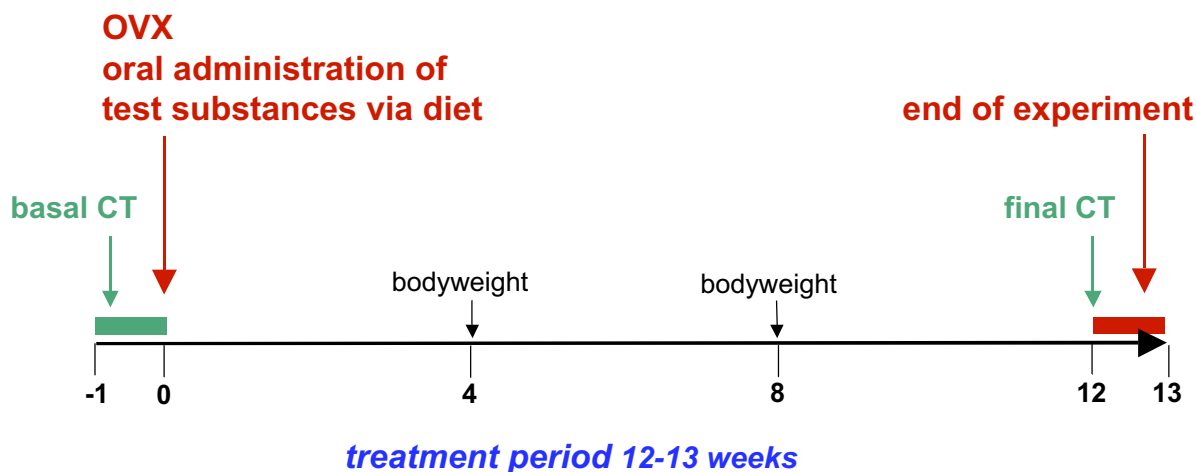


Figure 1-5: Time schedule of the long term treatment.

### Dose selection

Careful choice of dosage is required especially for this study design because the exposure level of the animal should provide a proper relation to human exposure levels. Two doses were selected for each substance to detect a possible switch from organ-selective to non-selective estrogenic effects.

The E2 doses were supposed to reflect the shift from the physiological into the supraphysiological range of treatment by factor 4.  $17\beta$ -Estradiol was administered as its ester  $17\beta$ -Estradiol-3-benzoate (E2B) to improve the low oral bioavailability of E2 and to reduce the liver load (Schleicher *et al.* 1998). The doses were chosen according to experiences and standard protocol established in the laboratory (Seidlova-Wuttke *et al.* 2003a; Seidlova-Wuttke *et al.* 2004).

There are numerous publications concerning dose and effective serum levels of GEN. The doses were chosen according to an overview of human and rat serum levels of GEN with the corresponding dosages (Doerge *et al.* 2002a). Thus the low dose is representing the exposure to GEN through the Asian diet or by a “regular” nutritional supplement, while the high dose correlates to a strong soy “cancer preventing” supplement intake.

A dose-dependent selectivity of effects could be expected by the 10 fold difference between the doses.

Dosages for RES and 8PN were set equimolar to GEN to enable direct comparison of final effects among the three test compounds.

RES is also well described and the resulting dosages lay within the range in literature. They also appeared reasonable considering the rule of thumb factor 5-10 between rat and human dosage. Nutritional supplements are offered in concentrations ranging from 5-10mg/day, which is claimed to be “entirely safe” for human use (Turner in (Haneke 2002)), to up to 100mg/day, which is roughly equivalent to the consumption of 5-10l red wine (Fremont 2000).

The analysis of a breast enhancement product revealed a daily dose of 100-150µg of 8PN (Coldham *et al.* 2001), that might be achieved with 1-2l of beer which can hold up to 100µg/l (Milligan *et al.* 1999). But hop is already identified as target for biotechnological modification aiming at increased 8PN levels for pharmaceutical production (Stevens *et al.* 2004). Concerning 8PN only 2 publications were found where it was administered to animals. In an adult ovx mouse uterotrophic bioassay approx. 0,5µg/g food did not evoke uterotrophic response (Coldham *et al.* 2001). Subcutaneous application to rats showed effects on uterus and bone after 2 weeks treatment with 30mg/kg BW (Miyamoto *et al.* 1998). Additional information contributed a poster displayed at the Summer School of Endocrinology 2003 (German Endocrine Society), where a dose of 10mg/kg BW s.c. for 3 days was tested in rats and exerted estrogenic changes in uterus (data published in the meantime (Diel *et al.* 2004)). Estimating a resorption rate between 10 and 40% for oral application, the 2 doses calculated in the equimolar approach should elicit responses in uterus and bone and allow detection of potential organ-selectivity.

		daily dose (mg) per kg bw			
<b>Control</b>	<b>ovx</b>	-			
	<b>intact</b>	-			
<b>Estradiolbenzoat</b>	<b>low</b>	0,19	→	physiological level	supraphysiological level
	<b>high</b>	0,75			
<b>Genistein</b>	<b>low</b>	6	→	Asian diet / regular nutritional supplement	high dose soy „cancer supplement“
	<b>high</b>	60			
<b>Resveratrol</b>	<b>low</b>	5	→	} equimolar to genistein dosages	
	<b>high</b>	50			
<b>8-Prenylnaringenin</b>	<b>low</b>	7,5	→		
	<b>high</b>	75			

Figure 1-6: Dosages applied in the long term experiment.

### Criteria for action in the HPG- and HPT-axes and related organs

The following overview compiles the main parameters selected for investigation. They are related to the modulation of gonadal and thyroid axes and potentially influenced by the activity of GEN, RES and 8PN under this long term treatment.

Table 1-1: Endpoints of potential endocrine activity of the test compounds and variables for investigation after long term treatment.

Endpoint	Morphology	Histology	Gene expression	Blood
<b>Pituitary</b>			LH $\beta$ , ER $\alpha/\beta$ TSH $\beta$	LH, FSH TSH Prolactin
<b>Uterus</b>	Weight	Uterine epithelium Lamina propria stromal cells	IGF1, C3, PR, ER $\alpha/\beta$	
<b>Vagina</b>		Vaginal epithelium Cornification		
<b>Liver</b>	Weight		IGF1, ER $\alpha/\beta$	Cholesterol, HDL, LDL Triglycerides
<b>Bone</b>	Trabecular density Bone mass Strength strain index			Osteocalcin RatLaps
<b>Thyroid</b>	Weight	Density of follicular cells Size and shape of colloids Thyroglobulin content	IGF1, ER $\alpha/\beta$	T3/T4, free and total Calcitonin

modified from (Diel *et al.* 2002)

ER $\alpha$  and ER $\beta$  vary in tissue distribution (Couse *et al.* 1997; Dechering *et al.* 2000) and both affect each others function when co-expressed, though underlying mechanisms are not completely understood yet (Matthews *et al.* 2003). The test compounds elicit different binding preferences for ER $\alpha$  and ER $\beta$ , which has become favorable in the development of SERMs (Katzenellenbogen *et al.* 2001). Therefore, both subtypes were assessed in all organs subjected to gene expression analysis.

Most important targets within the HPG-axis and most closely related to E2 action are the pituitary, uterus and vagina. Besides LH also FSH regulates the reproduction cycle together with the ovarian hormones E2 and progesterone. Prolactin was analyzed, since its release is influenced by E2 and T3 (DiPippo *et al.* 1995).

Uterus weight is the classical parameter of estrogenicity in the rodent uterotrophic assay (Odum *et al.* 1997; Kanno *et al.* 2001). Insulin-like growth factor-1 (IGF1), a protein belonging to the complement family (C3) and the progesterone receptor (PR) are important marker genes for expressional profiling in the uterus (Diel *et al.* 2000; Klotz *et al.* 2002).

Distinct alterations in uterine and vaginal histology are signaling estrogenic activity as well and help to detect very subtle influences (Diel *et al.* 2000; Haschek *et al.* 2001).

In the liver E2 strongly influences the important metabolites cholesterol, HDL, LDL and triglycerides (Tikkanen 1996), while liver weight is typically included for toxicological reasons.

For analysis of osteoporotic bone condition trabecular density is the main parameter. Together with bone mass and bone strength it can easily be determined by quantitative computer tomography (qCT) (Wronski *et al.* 1988). All three are modulated by both, E2 and T3 (Broulik *et al.* 2003). Information about skeletal remodeling is obtained from serum levels of osteocalcin and RatLaps, that are activity markers of osteoblasts and osteoclasts, respectively (Rosenquist *et al.* 1998; Young 2003).

Besides TSH and free and total thyroid hormones a closer inspection of the HPT-axis should include thyroid weight and histopathological assessment (DeVito *et al.* 1999; O'Connor *et al.* 1999). The thyroid is a target organ of E2 action also. ER were reported present in calcitonin secreting cells (Naveh-Many *et al.* 1992). Calcitonin is responsible for the calcium homeostasis in blood, that it maintains by decreasing calcium mobilization from the bone (Zaidi *et al.* 1993b).

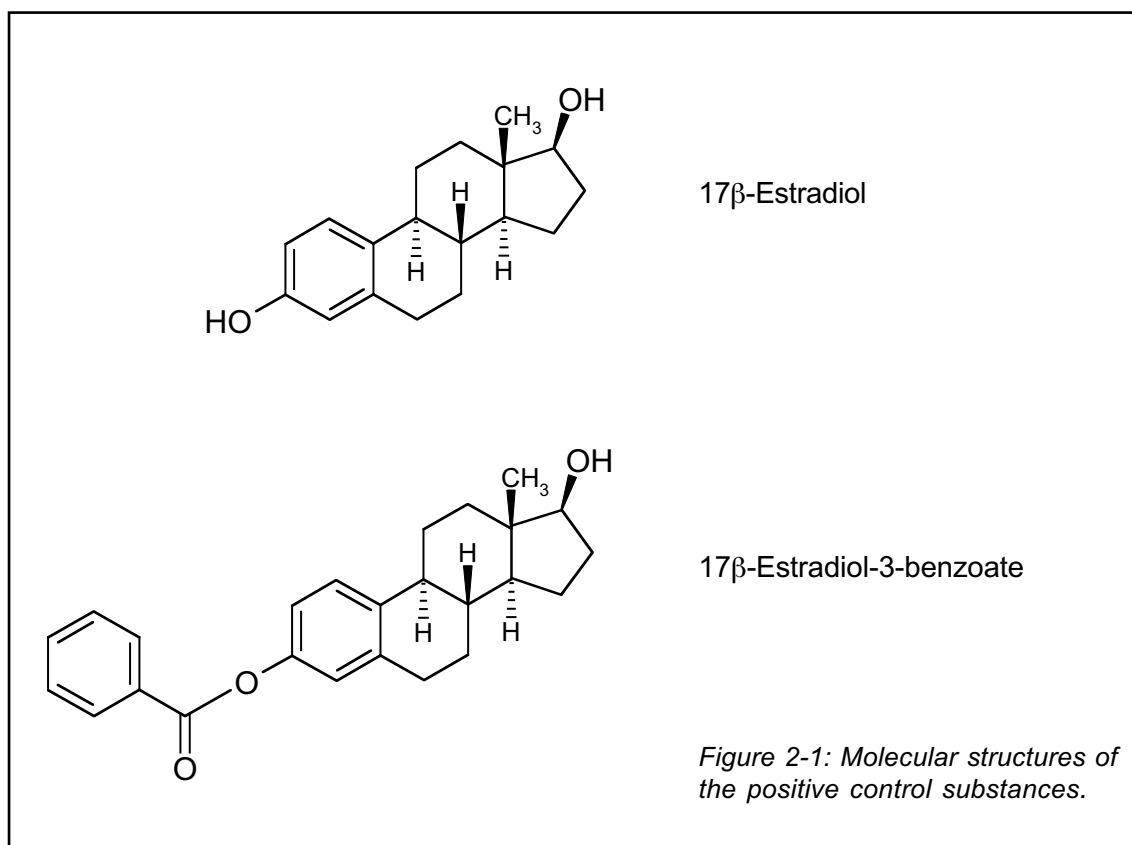
## 2 Material and Methods

### 2.1 Test compounds

Material Safety Data Sheets are available for E2 and E2B and require personal protection measures (classification as toxic and potential carcinogens). MSDS for GEN are also recommending care (classification as harmful). Since few data exist for RES and barely any for 8PN concerning personal risk, all substances were handled with the same appropriate precaution. Certificates of analysis by the manufacturers are attached.

#### 2.1.1 Positive controls

Chemical names                      17 $\beta$ -Estradiol, 17 $\beta$ -Estradiol-3-benzoate  
 Chemical family                    steroids



	<u>17<math>\beta</math>-Estradiol (E2)</u>	<u>17<math>\beta</math>-Estradiol-3-benzoate (E2B)</u>
Molecular weight	272,4 g/mol	376,5 g/mol
Molecular formula	C <sub>18</sub> H <sub>24</sub> O <sub>2</sub>	C <sub>25</sub> H <sub>28</sub> O <sub>3</sub>
CAS#	50-28-2	50-50-0

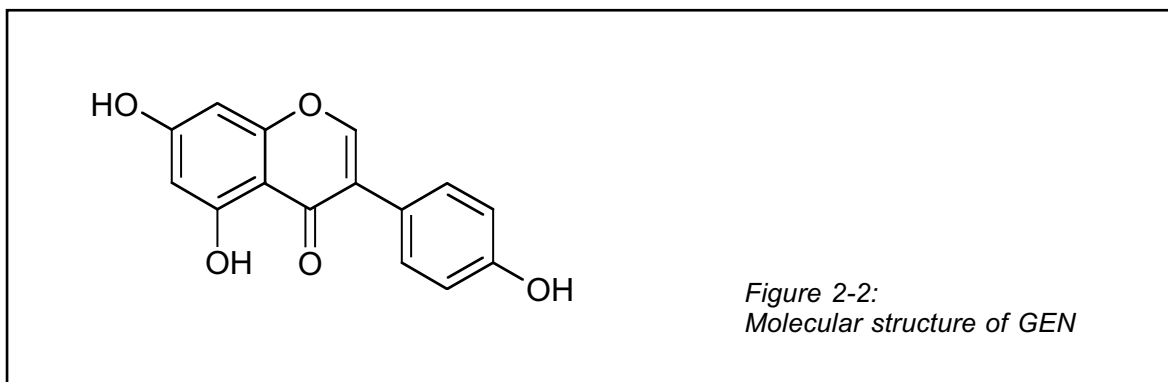
Table 2-1: Information on the material used (positive controls)

Origin	Supplier	Batch no.	Content	Method	Storage	Experimental use	
E2	Synthetic	Sigma E-8875	121K1448	100%	HPLC	RT	Single dose treatment, cell culture
E2B		Sigma E-8515	072K0996	98,50%			Long term treatment

### 2.1.2 Genistein

Chemical name or synonym 4',5,7-trihydroxyisoflavone

Chemical family flavonoids, isoflavones



Molecular weight 270,2 g/mol

Molecular formula  $C_{15}H_{10}O_5$

CAS# 446-72-0

Natural sources

- Soy beans, *Glycine max. L.*, Fabaceae (Setchell *et al.* 1987)
- Red clover herb and blossoms, *Trifolium pratense*, Fabaceae (Liu *et al.* 2001)

Table 2-2: Information on the material used (GEN)

Origin	Supplier	Batch no.	Content	Method	Storage	Experimental use
Synthetic	Chemos	SG20030618	> 98,5%	HPLC	2-8°C	Long term treatment, cell culture
	Roth	6x 5358965 2x 47256868 1x 46256767	> 95%	HPLC		Single dose treatment

### 2.1.3 Resveratrol

Chemical name or synonym *trans*-3,5,4'-trihydroxystilbene

Chemical family polyphenols, stilbenes

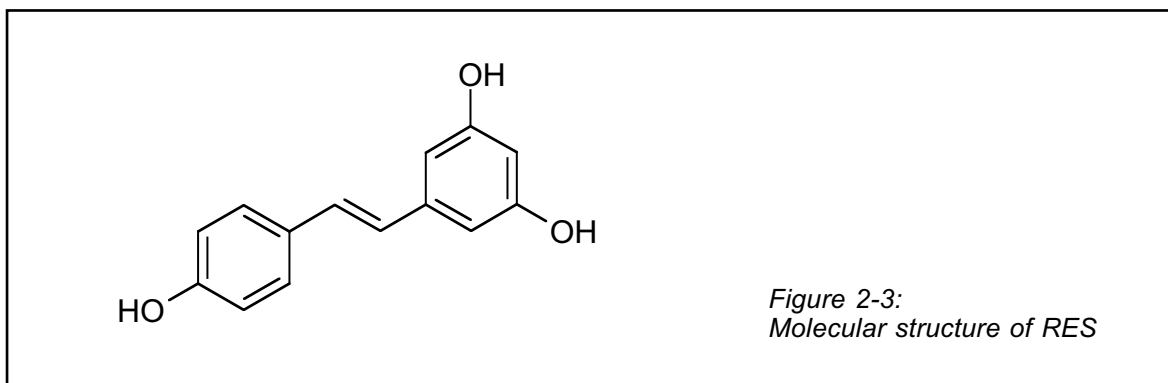


Figure 2-3:  
Molecular structure of RES

Molecular weight 228,2 g/mol

Molecular formula  $C_{14}H_{12}O_3$

CAS# 501-36-0

#### Natural sources

- Grape vines, leaves and berries, *Vitis vinifera*, Vitaceae (Daniel *et al.* 1999)
- Knotweed rhizome, *Polygonum cuspidatum*, Polygonaceae (Yang *et al.* 2001)

Table 2-3: Information on the material used (RES)

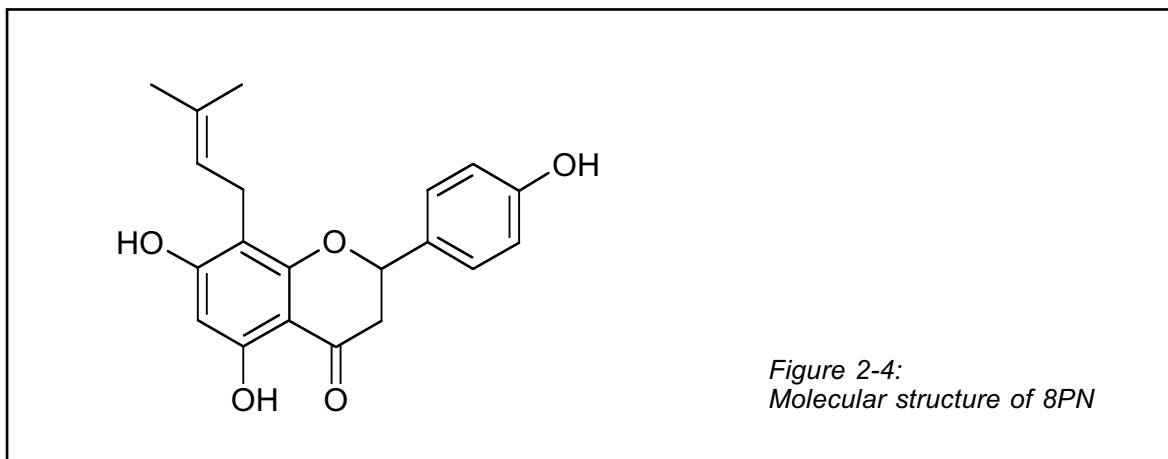
Origin	Supplier	Batch no.	Content	Method	Storage	Experimental use
Knotweed rhizome	Nabio Biotechnology, Shanghai	20030721	99,20%	HPLC	-20°C	Long term treatment
Sythetic	Sigma, München	111 K5206	100%	GC, TLC		Single dose treatment, cell culture

### 2.1.4 8-Prenylnaringenin

Chemical name or synonym 5,7,4'-trihydroxy-8-prenyl-flavanone

8-isopentenylnaringenin

Chemical family prenylflavonoids, flavanones



Molecular weight 339,4 g/mol

Molecular formula  $C_{20}H_{20}O_5$

#### Natural sources

- Female hop cones, *Humulus lupulus*, Cannabinaceae (Milligan *et al.* 1999)
- Heartwood of *Anaxagorea luzonensis*, Annonaceae (Kitaoka *et al.* 1998)

Table 2-4: Information on the material used (8PN)

Origin	Supplier	Batch no.	Content	Method	Storage	Experimental use
Synthetic	Schering AG, Berlin Prof. Hans Becker Dept. of Pharmaceutical Biology University of Saarbrücken	produced 11/2002 2003	99,9% rac. > 95% rac.	NMR	2-8°C	Long term treatment Single dose treatment, cell culture

The chemical synthesis of the material yielded a racemic mixture in both cases, which in the experiments was used as is. Both naturally occurring pure enantiomers (2S(-)8PN and 2R(+)-8PN) were separately tested *in vitro* and *in vivo*, where ER affinity and estrogenic activity only moderately differed (Schaefer *et al.* 2003).



## 2.2 Pituitary cell culture

### 2.2.1 Media

#### Spinner medium

500 ml	SMEM
7 ml	BSA 7,5%
5 ml	Hepes 1M
5 ml	Pyruvate
5 ml	Pen/Strep

#### Serum medium (with phenolred)

500 ml	DMEM
22,5 ml	FCS
40 ml	HS
5 ml	Pen/Strep
5 ml	Pyruvate

#### Permanent culture medium (w/o phenolred)

500 ml	DMEM
50 ml	FCS charcoal stripped
5 ml	Pen/Strep
5 ml	Pyruvate
5 ml	Glutamine

Details of reagents used for cell culture can be viewed in chapter 7 Fact sheets.

### 2.2.2 Procedure

#### Start of culture

For the primary culture anterior pituitaries of female Sprague-Dawley rats (200-250g bw) were removed after decapitation under CO<sub>2</sub> anaesthesia.

The freshly excised organs were immediately dropped into 50ml Spinner medium on ice.

Pituitaries were coarsely cut with scalpels and rinsed to remove erythrocytes. The pieces were incubated with Spinner medium containing Trypsin (5mg/ml) and DNase (both Sigma) at 37°C for 90min and triturated every half an hour. The cell suspension was passed through a cell strainer and digestion stopped by addition of serum containing medium. The suspension was centrifuged twice at 470g/10min to exchange the Spinner with serum medium. Cell number was determined in the Neubauer chamber and the suspension diluted to 250.000 cells/ml. 96-well plates were prepared with 250µl/well and cultured at 37°C, 10% CO<sub>2</sub> and 90% relative humidity for 4 days.

#### Incubation with test substances

Cells were washed and maintained under steroid-free conditions with permanent culture medium. Substances were added from concentrated stock solutions (10<sup>-2</sup>M) in EtOH.

The cells were incubated for 48h with final concentrations of  $10^{-6}$ M GEN, RES, 8PN, ICI 182,780 and  $10^{-9}$ M E2. After 45h half of the plates were stimulated for the last 3h with a mixture of GnRH (Sigma) and TRH (Bachem) in a final concentration of  $10^{-6}$ M. At the end of the incubation period the supernatants were removed and kept at  $-20^{\circ}\text{C}$  until measurement of LH and TSH production by RIA (see Serum analysis 2.7.1 Hormones p.44). 100 $\mu\text{l}$  remained in each well to perform a MTT-test to check cell viability.

	1	2	3	4	5	6	7	8	9	10	11
<b>A</b>	<b>Med.</b>	<b>LM</b>	<b>ICI</b>	<b>E2 -9M</b>		<b>GEN -6M</b>		<b>RES -6M</b>		<b>8PN -6M</b>	
<b>B</b>											
<b>C</b>											
<b>D</b>											
<b>E</b>											
<b>F</b>		<b>-6 M</b>	<b>-6 M</b>	<b>E2</b>	<b>E2 + ICI</b>	<b>GEN</b>	<b>GEN + ICI</b>	<b>RES</b>	<b>RES + ICI</b>	<b>8PN</b>	<b>8PN + ICI</b>
<b>G</b>											
<b>H</b>											

Figure 2-4: Treatment scheme of the 96 well plates.

### MTT-Test

With additional 10 $\mu\text{l}$  of MTT-solution (5mg/ml in PBS) cells were incubated for 1,5-2h to convert sufficient amounts of MTT. The reaction was stopped and fixed by 10 $\mu\text{l}$  Glutardialdehyde (12,5% in PBS) for 20 min. All supernatant was removed. 150 $\mu\text{l}$  0,04N HCl in isopropanol were pipetted into each well and plates were left on a shaker to enhance lysis of cells and dissolving of the blue precipitate. Optical density was determined at 550nm and a reference filter at 690nm with the TECAN Spectra Reader and Magellan-Software.

## 2.3 Animals

### 2.3.1 Ethical approval of experiments

All animal experiments were performed according to the general German animal welfare regulations and with legal approval no. 509.42502/01-36.03 by the district authorities Braunschweig. For premature study termination the following criteria were defined: Animals should be sacrificed, if they were seriously harmed or did not have the possibility to continue living without suffering or having severe pain. Animals found dead or sacrificed moribund were necropsied.

### 2.3.2 Strain and breeding

All experiments were performed with Sprague-Dawley rats provided by Fa. Winkelmann, Germany.

For the single dose and long term treatments animals were specifically bred in our premises: The parental generation was obtained January 2003 at the age of 2 months and permanently switched to soyfree diet.

Parents were mated for the first time 2 weeks upon arrival, and the litters were born between May 17<sup>th</sup> and 25<sup>th</sup>, 2003: Female animals entered the single dose treatment at the age of four month. After a second mating the litters were born between May 2<sup>nd</sup> and 6<sup>th</sup>, 2003: Female animals entered the long term treatment at the age of four months.

All pups were raised and kept under soyfree conditions and are designated as a "generation F0".

### 2.3.3 Husbandry

Facilities	Experimental animal housing, University of Göttingen
Conditions	20°C temperature, 55% rel. humidity, 12h light/dark cycle
Accommodation	5-6 animals per cage (Makrolon® IV)
Diet and water	ad libitum, community tap water

Cages of the animals were changed every 3 days.

### 2.3.4 Animal diet

#### Regular diet

Animals were kept constantly on a soyfree formulation of diet, ssniff SM R/M, 10 mm supplied by ssniff Spezialdiäten GmbH, Soest, Germany.

The composition is given as an attachment (7 Fact sheets).

#### Preparation and handling of batches of test compounds

The supplemented food was prepared by mixing the test substances with that formulation to homogeneity before the process of pelleting. All batches were prepared 1 week prior to the start of the experiment. Each bag of food was labeled with the respective tag of the group name and number, which was also used for the cages of the test animals.

All batches had an expiry date of 4 months after manufacturing.

30g of each batch were kept at -20°C, if further analysis is required.

Special safety measures were taken to prevent inhalation of dust or percutaneous absorption whenever handling the food pellets or the cages containing it. Food was

stored separately, and single use gloves, hair and mouth cover, coat were used for personal protection.

<b>2</b>	<b>CON</b>	<b>soyfree</b>	batch no.	6093223
<b>5</b>	<b>GEN low</b>	<b>0,1 g/kg food</b>	batch no.	9073536 / 1
<b>7</b>	<b>RES low</b>	<b>0,084 g/kg food</b>	batch no.	9073536 / 3
<b>9</b>	<b>8PN low</b>	<b>0,126 g/kg food</b>	batch no.	9073536 / 7
<b>11</b>	<b>E2B low</b>	<b>4,3 mg/kg food</b>	batch no.	6683339
<b>6</b>	<b>GEN high</b>	<b>1 g/kg food</b>	batch no.	9073536 / 2
<b>8</b>	<b>RES high</b>	<b>0,84 g/kg food</b>	batch no.	9073536 / 4
<b>10</b>	<b>8PN high</b>	<b>1,26 g/kg food</b>	batch no.	9073536 / 8
<b>1</b>	<b>E2B high</b>	<b>17,3 mg/kg food</b>	batch no.	9073536 / 9

Figure 2-5: Overview of concentrations of test compounds and batch no.s of manufactured food.

### Storage

The total amount of each supplemented food was always delivered in 2 bags.

The first bag was kept at room temperature for the twice weekly feeding, the second at -20°C until use. Depending on food intake one bag normally lasted for approximately 7-11 weeks of the experimental time.

### Stability

For stability testing content of the test compounds in the animal food was determined along the treatment time by HPLC-UV developed for the quantitative detection of each test compound (see HPLC 2.6.2 Analysis of animal diet p.37).

### 2.3.5 Ovariectomy

At the age of 4 months animals were ovariectomized. They were briefly exposed to CO<sub>2</sub> until they fell unconscious and then anaesthetized via i.p. injection of Ketamin (Hostaket®, Hoechst) und Xylazin (Rompun®, Bayer). Dose per kg bw was 7,5mg Xylazin and 62,5mg Ketamin. In case further anaesthesia was required, animals received an additional 100mg per kg BW of Ketamin only. For eye protection a hydrating gel (Thilo-Tears®, Alcon) was applied. The flanks were shaved and disinfected before abdominal incision. Then the ovaries were prepared, ligated beneath the tubes with sterile resorbable catgut (3 metric, Ethicon) and resected with a scalpel. After repositioning of the uterus horn in the abdominal cavity, the tunica muscularis was sutured with catgut, the skin closed with Michel suture clamps® (Martin Medizintechnik). To prevent dehydration effects animals were subcutaneously administered 5ml of isotonic NaCl-solution (Braun). To shorten

recovery time a dose of 0,5mg/kg BW of Atipamezol-HCl (Antisedan®, Pfizer) was injected intraperitoneally. Animals were monitored until reawake.

## 2.4 Single dose treatment

The experiment was performed with female rats (raised soyfree as described in chapter 2.3.2 Strain and breeding p.26), who were ovariectomized 4 weeks before at the age of 4 months.

The realization of the experiment required 2 days, with jugular vein surgery on the first and actual treatment on the second day. Treatment time was 6 h.

Table 2-4: Timing scheme of experimental procedure on the day of treatment.

<b>treatment time</b>		<b>-1h</b>	<b>-0,5h</b>	<b>0</b>	<b>0,5h</b>	<b>1h</b>	<b>2h</b>	<b>3h</b>	<b>4h</b>	<b>6h</b>
<b>daily schedule</b>	<b>7.00</b>	<b>8.00</b>	<b>8.30</b>	<b>9.00</b>	<b>9.30</b>	<b>10.00</b>	<b>11.00</b>	<b>12.00</b>	<b>13.00</b>	<b>15.00</b>
acclimatization	1hour	1	2	3	4	5	6	7	8	<b>Sacrifice</b>
		serial samples								trunk blood

### 2.4.1 Allocation and dosage

Animals received test compounds per gavage. Substances were suspended in a 0,5% solution of carboxymethylcellulose sodium salt (viscosity of 1% aqueous sol. at 25°C: 1500-3000cps, Sigma C5013 batch 65H0033), while plain solution was used for control animals.

Table 2-5: Treatment groups of the single dose experiment and dosages for intragastric application of the test compounds.

	Dosages (mg)		no. of animals
	per animal	per kg bw	
<b>CON</b>			<b>6</b>
<b>GEN</b>	<b>50</b>	<b>158,5</b>	<b>8</b>
<b>RES</b>	<b>50</b>	<b>153,0</b>	<b>8</b>
<b>8PN</b>	<b>50</b>	<b>161,4</b>	<b>9</b>
<b>E2</b>	<b>5</b>	<b>15,5</b>	<b>8</b>

### 2.4.2 Jugular vein catheterization

The catheter was implanted to allow repeated withdrawal of blood samples from the animals in order to minimize manipulation induced stress of the animals. (Shah *et al.* 1977; Bakar *et al.* 1983; Vachon *et al.* 2001; Thrivikraman *et al.* 2002)

### **Preparation of catheter**

A catheter consists of 20cm of silicon tubing (Medical grade, Dow Corning, ID 0,5mm, OD 0,9mm), and a teflon shield (Perthese® silicon sheeting, LPI) of approx. 0,5x1cm fixed to it with silicon glue (Elastosil®, Wacker-Chemie) at one end.

### **Implantation**

The rats received the indwelling catheter in the jugular vein under anaesthesia with isoflurane. After lateral incision of the neck skin the jugular vein was prepared, the catheter was inserted into the vein with a bent 22G cannula (Sterican®, Braun), that was removed through the muscle layer, while the catheter remained inside the lumen. Its flux was tested with heparin-solution (50 I.E. Liquemin®, Roche, with physiological saline). Suturing subcutis with resorbable surgical silk (3 metric, Ethicon), the catheter was fixed with the teflon shield at the proximate site. The tubing was passed subcutaneously towards the back of the animal and stored in a previously prepared bag for easy access to the tubing at the back neck of the animal. Skin was sutured with nonresorbable surgical sewing material (Supramid 2/0 metric, Braun Aesculap). For recovery the animals remained in single cages overnight. The next day the catheter was extended with a 1 ml syringe.

### **Blood withdrawal**

The next morning the catheter was extended with a 1 ml syringe and tubing kept free of clogging blood by rinsing with 25 I.U. heparin solution. Animals were left for an accommodation period before withdrawing any blood. Sample volume was 300µl and always substituted with the same volume of physiological saline.

### **2.4.3 End of experiment**

Since treatment lasted 6h and the dose was administered around 9 a.m., animals were sacrificed between 15 and 16 p.m. Animals were decapitated with a guillotine under CO<sub>2</sub> anaesthesia.

Besides the serial samples drawn during treatment time, trunk blood was collected after decapitation. Uteri were prepared for weighing. Serial and trunk blood samples were centrifuged (3000g, 20min) and the serum stored at -20°C for further analysis.

### **2.4.4 Parameters**

Body weight before surgery

Uterus wet weight

LH, TSH in serial samples

LH, TSH, total and free T3 and T4 in trunk blood

Levels of applied substances in trunk blood

- E2 in CON, E2 groups by RIA
- GEN, RES, 8PN by HPLC-UV

## 2.5 Long term treatment

The experiment was performed with female rats (raised soyfree as described in chapter 2.3.2 Strain and breeding p.26), 4 months of age. Treatment started with ovariectomy and lasted for 12-13 weeks. The test substances were administered orally via their pelleted food, that was manufactured in the intended concentration. An age-matched intact group was included in the final CT measurement, sacrifice and obtaining of the final parameters.

*Table 2-6: The table gives an overview of the treatment schedule. Group 11 and intact animals ended the study with a delay due to organizational and technical reasons.*

*Female S.D. rats (age in weeks)  
F0 soyfree*

GROUP	treatment week	132 animals treated		12 animals intact
		1-10	11	
Basal CT	-1/0	17	20	
Ovx	0	18	20	
Final CT	12	30	32	31
Sacrifice	12/13	31	32	32

### 2.5.1 Allocation and dosage

The treatment groups consisted of 12 animals in all, 2 cages of 6 animals each.

*Table 2-7: Treatment groups, their abbreviation and group no. and respective dosage of test compounds.*

Treatment	label	group	daily dose (mg/kg bw)
<b>Control ovx</b>	CON	2	-
<b>Estradiolbenzoate</b>	E2B low	11	0,19
	E2B high	1	0,75
<b>Genistein</b>	GEN low	5	6
	GEN high	6	60
<b>Resveratrol</b>	RES low	7	5
	RES high	8	50
<b>8-Prenylnaringenin</b>	8PN low	9	7,5
	8PN high	10	75

To identify the individual animals, they received a subcutaneous implant of a transponder microchip after the basal CT, while still under anaesthesia.

To achieve an even distribution within the groups, animals were allocated according to their day of birth (May 2<sup>nd</sup>-6<sup>th</sup>, 2003).

## 2.5.2 Measurements during treatment period

### Body weight development

The body weight of each rat was measured at basal CT, ovariectomy, 4 and 8 weeks of treatment, at urodynamics after 11 weeks, at final CT after 12 weeks and before sacrifice.

### Food and water consumption

These parameters were determined cagewise, accounting for 6 animals:

- intake of food twice weekly
- intake of water three times a week

### Bone mineral density

Bone mineral density of metaphysis of the tibia and of a vertebral body was measured before (basal CT) and after 12 weeks of treatment (final CT) under anaesthesia with isoflurane (Forene®, Abbott). (See 2.10 Computer tomography p.52)

### Vaginal smears

Vaginal smears were taken with a small cotton swab during the time of the final CT-scan after 12 weeks of treatment.

## 2.5.3 End of experiment

Of course the animals of this 3 months study were valuable for various other investigative aspects. Therefore more biological samples and parameters were obtained than required for the outlined variables and were also available for collaborating partners of the EURISKED consortium.

Animals were decapitated with a guillotine under CO<sub>2</sub> anaesthesia. The animals were sacrificed between 8 and 12 a.m. to ensure a negative feedback action of estradiol or test compounds on gonadotropin secretion. To exclude any effect of time of death between the groups, they were evenly distributed over the whole time frame.

Trunk blood was collected after decapitation and the samples were obtained as displayed in *able 2-8*:

Organs		- 70°C	Formalin 10%
Pituitary		X	
Thyroid		X	
Uterus	seperate horns	X	X
Vagina			X
Liver	middle lobe	X	X



All specimen for molecular biology analysis were snap-frozen in liquid nitrogen within 3-5 minutes to minimize degradation of RNA, then stored at -70°C. Selected tissues were immediately fixed with 10% formalin solution for further histological analysis.

The blood samples were centrifuged (3000g, 20min) and serum was stored at -20°C. (see 2.7 Serum analysis p.44)

#### 2.5.4 Final parameters

##### Organ weights

All organs were weighed immediately after preparation in the following sequence:

Thyroid - Uterus - Liver

##### Serum parameters

Levels of applied substances:

E2 in CON, E2B low and high, intact groups by RIA

GEN, RES, 8PN by HPLC-UV

Hormonal parameters:

Pituitary hormones LH, FSH, TSH, prolactin

Thyroid hormones total and free T3 and T4, calcitonin

Metabolic parameters:

Cholesterol, triglycerides, HDL, LDL, leptin

Osteocalcin, RatLaps

##### Molecular biology

Table 2-9 gives an overview of tissues and determined genes:

	ER $\alpha$	ER $\beta$	$\alpha$ subunit	LH $\beta$	TSH $\beta$	IGF1	C3	PR
Pituitary	X	X	X	X	X			
Uterus	X	X				X	X	X
Thyroid	X	X				X		
Liver	X	X				X		

##### Histology

Morphological changes in

- Uterus
- Vagina
- Thyroid

## 2.6 HPLC

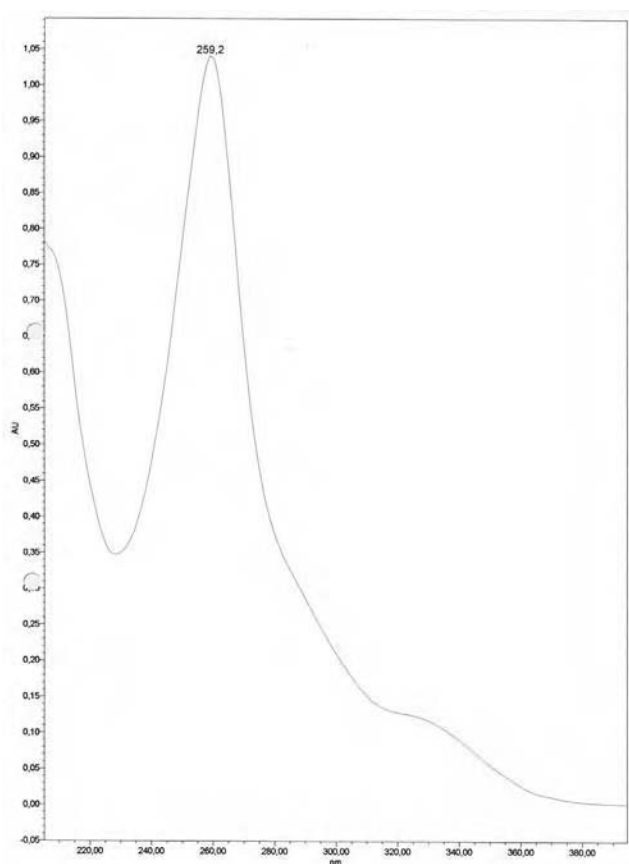
All analyses were performed with RP-18 material (5 $\mu$ m particle size) and 250x4,0/4,6mm columns. Details of materials can be viewed in chapter 7 Fact sheets.

### 2.6.1 Pure compounds

The three test substances were identified by comparison of chromatograms with material on stock in the lab. Peaks other than respective substance detected in the HPLC were not attributed to defined compounds.

A sensitivity test was performed for each substance dissolved in EtOH (10<sup>-3</sup>M to 10<sup>-8</sup>M) and yielded a quantification limit (signal/noise 10:1) with sufficient linearity at 5\*10<sup>-8</sup>M for the three of them. The detection limits (S/N 3:1) lay between 1\*10<sup>-8</sup>M and quantification limit.

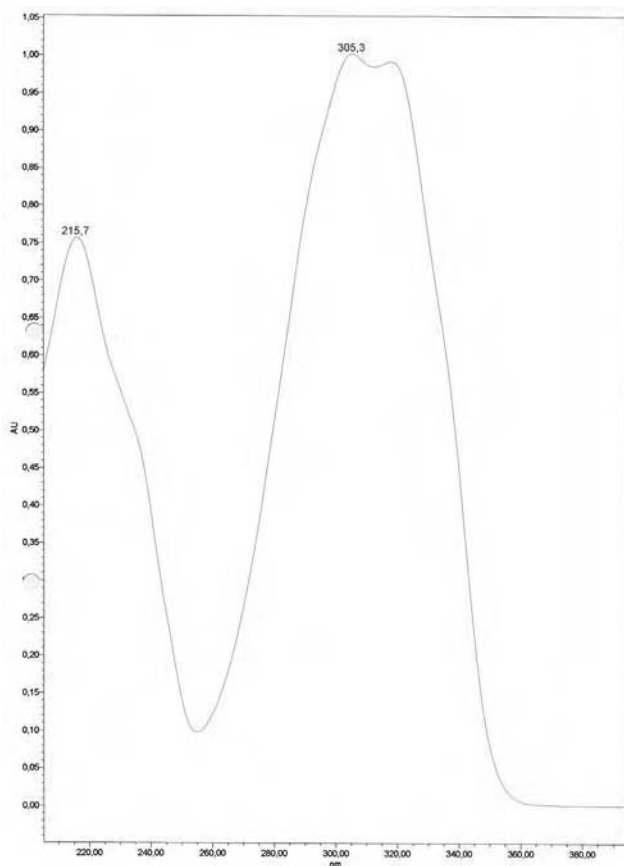
#### 2.6.1.1 UV-spectra



Genistein

Figure 2-6: UV-spectrum of GEN (in EtOH)

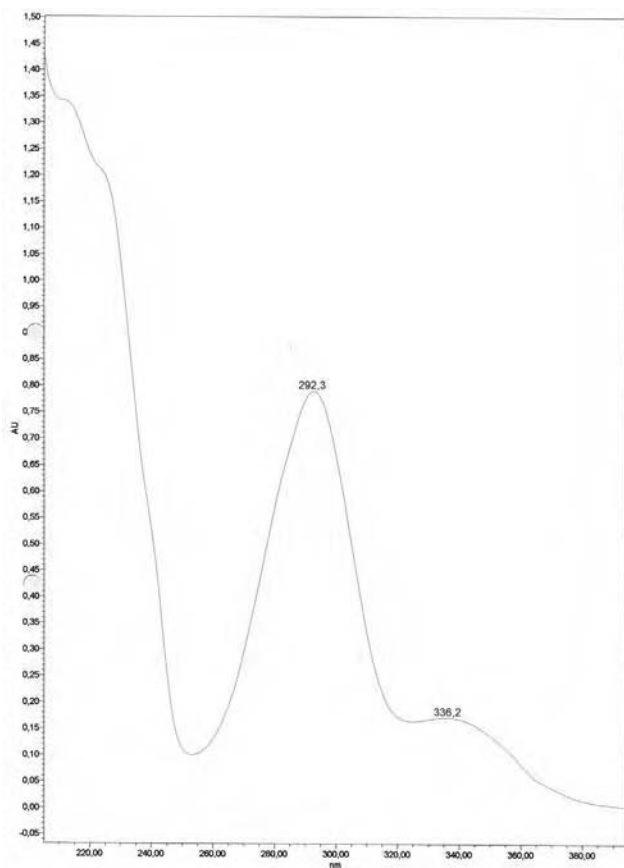
$\lambda$  max 259 nm,  $\lambda$  min 225 nm



Resveratrol

Figure 2-7: UV-spectrum of RES (in EtOH)

$\lambda$  max 216,305 nm,  $\lambda$  min 255 nm



8-Prenylaringenin

Figure 2-8: UV-spectrum of 8PN (in EtOH)

$\lambda$  max 215,292 nm,  $\lambda$  min 257 nm

### 2.6.1.2 HPLC conditions

**Column** 250x4,0mm

#### Autosampler

Injection volume 10 $\mu$ l

Sample loss 30 $\mu$ l

#### UV-detector

GEN 260nm

8PN 220nm

RES 305nm

#### Solvents (HPLC-grade, J.T.Baker)

A Water containing 0,085% o-phosphoric acid (approx. pH 2)

B Acetonitril

#### Gradients (Table 2-10)

Flow 1ml/min

min	A %	B %
0	70	30
15	25	75
16	0	100
21	0	100
22	70	30
29	70	30

retention time

**GEN** 9,0 min  
**8PN** 13,5 min

min	A %	B %
0	80	20
20	75	25
21	0	100
26	0	100
27	80	20
32	80	20

retention time

**RES** 10,2 min

### 2.6.1.3 Chromatograms

Representative chromatograms of the three test compounds in 100% ethanol are shown in the following figures. They were obtained at the respective wavelength and with the gradient described in the previous paragraph on HPLC conditions.

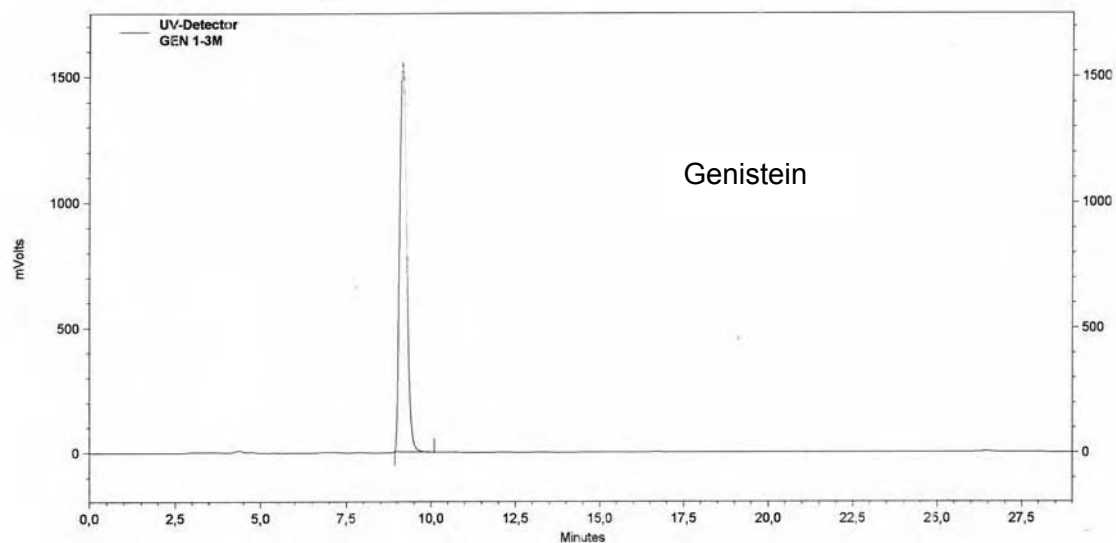


Figure 2-9: HPLC-UV chromatogram of GEN at 260 nm, retention time 9,0 min.

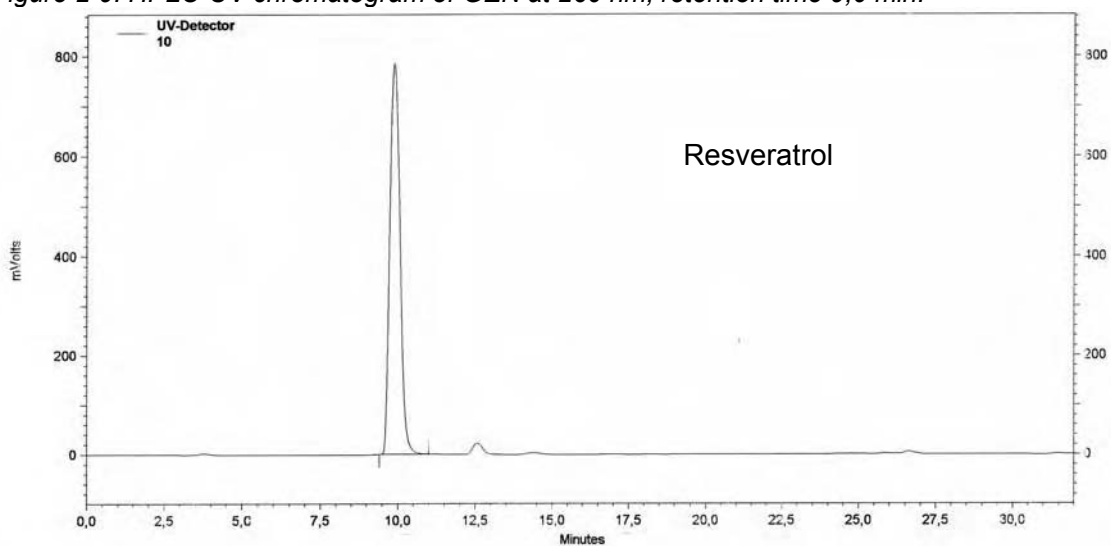


Figure 2-10: HPLC-UV chromatogram of RES at 305 nm, retention time 10,2 min.

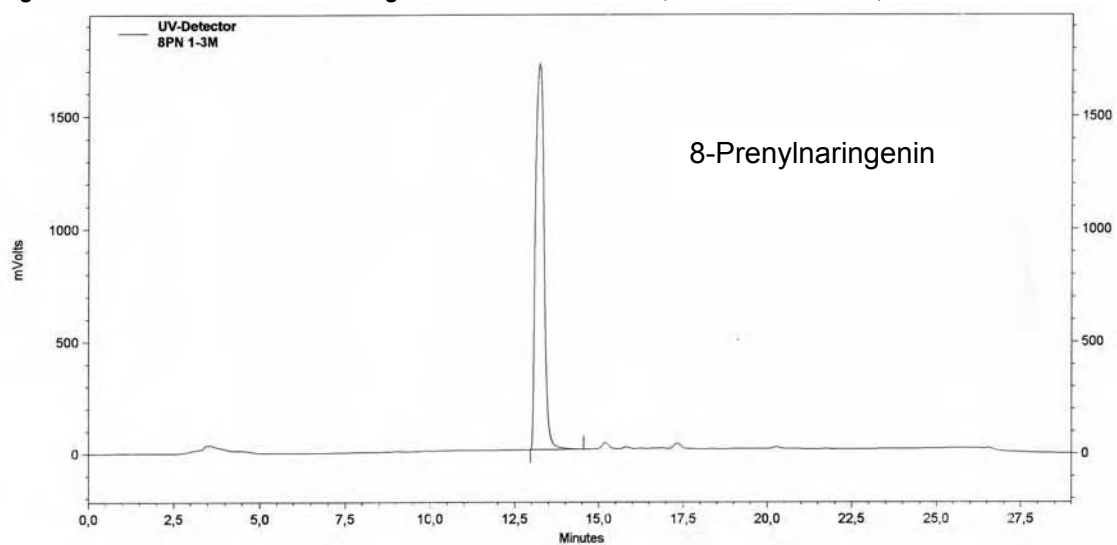


Figure 2-11: HPLC-UV chromatogram of 8PN at 220 nm, retention time 13,5 min.

## 2.6.2 Analysis of animal diet

Food pellets were pulverized with an electric mill (SpeedyProPlus, Krups) and 10g were extracted with 30ml of EtOH 99% stirring overnight. First the suspension was filtrated through a 595 1/2 folded filter (Schleicher&Schuell), then 1ml of the clear yellow fluid filtered again for HPLC-UV through a Minifilter (Millex®-HV, Millipore) with 45µm pore size.

### 2.6.2.1 HPLC conditions

As described under 3.5.2 Pure compounds, with the following exception:

#### UV-detector

RES 260nm

- selected for codetermination of a food component eluting very close before

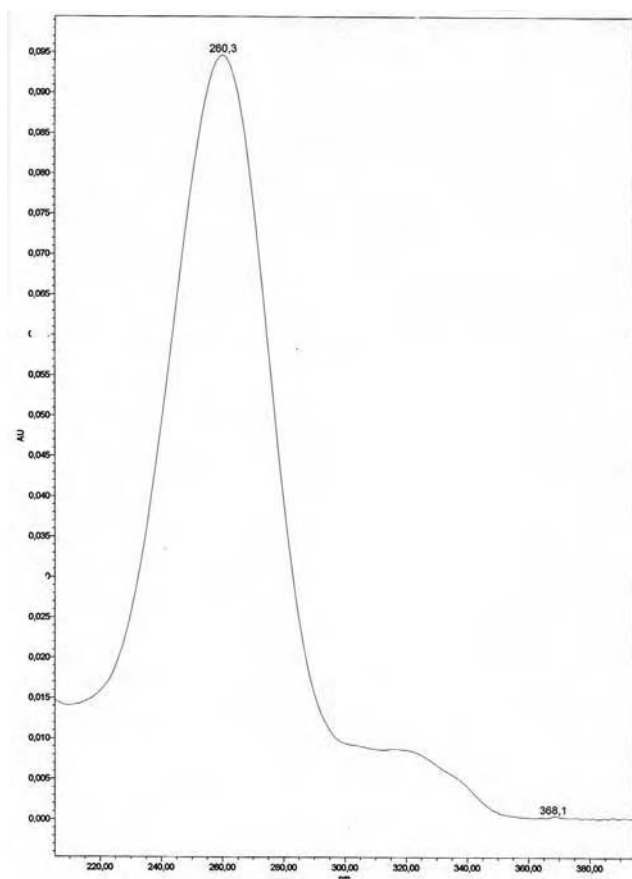


Figure 2-12: UV spectrum of a food component that interferes with the chromatographic detection of RES after the ethanolic extraction.

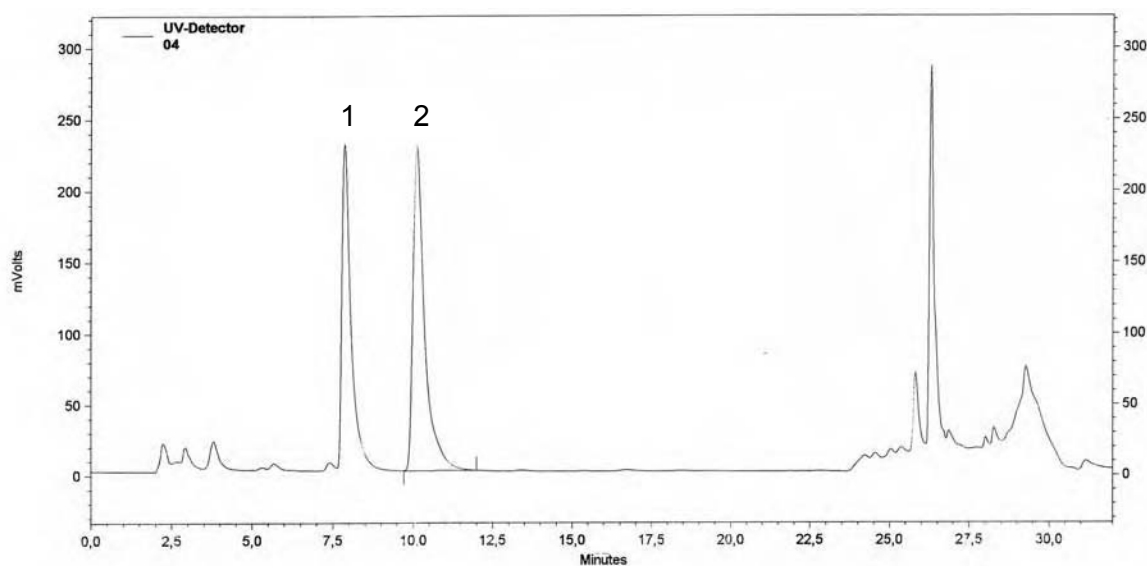


Figure 2-13: Exemplary chromatogram of baseline separation of an interfering food component (1) and RES (2) obtained at 260nm.

#### 2.6.2.2 Recovery of the analytes from food

Soyfree food pellets were pulverized with a rotor mixer and the powder added by steps to the required amount of test compound. Then the mixture was extracted as described above. Two triplicate spike experiments were performed with each compound in the two respective doses (intra-assay variability) and double injected (inter-assay variability).

#### 2.6.2.3 Stability of analytes in food

The content of the three test substances was determined at the start and the end of an opened bag, respectively at the end of the study. The first bag of food pellets lasted for 7-10 weeks depending on consumption by the animals.

#### 2.6.2.4 STD-curves for determination of food contents

Dilutions of the test substances in ethanol were used to construct calibration curves and their concentration range was selected to match with the low and high doses (Table 2-11). The linearity of the method in the calibrated range was sufficient with  $R^2$  exceeding 0,995 (Table 2-12).

Table 2-11: Distribution of STD-points

	GEN	RES	8PN	STDs				
<i>MAX conc. expected (µg/ml)</i>								
<b>low</b>	33,3	28	42	10	20	30	40	50
<b>high</b>	333	280	420	100	200	300	400	500

Table 2-12: Resulting standard curves for food analysis

GEN	RES	8PN
$y = 0,0345x + 0,0818$	$y = 0,0331x + 0,128$	$y = 0,0473x + 0,6751$
R2 = 0,9997	R2 = 0,9993	R2 = 0,9949

### 2.6.3 Analysis of serum

To recover the substances from serum in detectable amounts for HPLC-UV, it was necessary to regain the free molecule from glucuronidated and sulfated metabolites. Acid hydrolysis and precipitation of protein in the serum did not prove to be sufficient in this system. The following method allowed quantification of total serum levels achieved with the low doses.

#### 2.6.3.1 Enzymatic hydrolysis of metabolites

Helix Pomatia  $\beta$ -Glucuronidase Type H1 (Sigma cat# G-0751)

Glucuronidase activity            min 300.000 U/g

Sulfatase activity                 min 10.000 U/g

Samples were prepared in 24-well plates.

500µl serum were diluted with 500µl NH<sub>4</sub>acetate buffer (pH 5,0) containing 1mg  $\beta$ -Glucuronidase/sample.

After mixing carefully on a shaker, the plates were incubated for 12 to 14 hours at 37°C. The next day they were removed and let cool down to room temperature at least 30 min prior to extraction.

#### 2.6.3.2 Extraction

The Strata X solid phase extraction method with a polymeric sorbent was chosen for extraction. Columns of a 60mg bed mass/3ml washing and elution volume (8B-S100-UBJ, Phenomenex) were used according to the standard protocol suggested in the user's guide. Methanol was substituted with ethanol, because the analysis of animal diet was



already performed with EtOH as solvent and parallel extraction with both alcohols gave comparative results:

Conditioning	3ml	EtOH 100%
Equilibrating	3ml	deionized water
Loading	1ml	sample
Washing impurities	3ml	EtOH 5%
Drying	30sec	vacuum
Eluting analyte	3ml	EtOH 100%

### Solvent removal

The sample of the last step was collected in a glass tube and evaporated to dryness without heating in a vacuum centrifuge (Speedvac) which took about 5-6h.

### Reconstitution

Analytes were reconstituted with 100µl EtOH 100%, rinsing the interior of the glass tube and vortexing well. Then the samples were transferred to HPLC-vials with 300µl micro-inserts using a 1ml syringe and filtering through Millex<sup>®</sup>-HV Syringe Driven Filter Unit (Low Protein Binding Durapore PVDF membrane 0,45µm/4mm, Millipore, Cat.No. SLHV R04 NL) to remove remaining protein pollutants.

#### 2.6.3.3 External and internal standards

4-Methyl-umbelliferone (4MU), its glucuronidated and sulfated form were used as standards. 4MU was always detected simultaneously with the fluorescence detector.

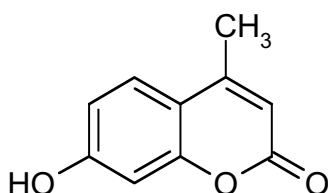
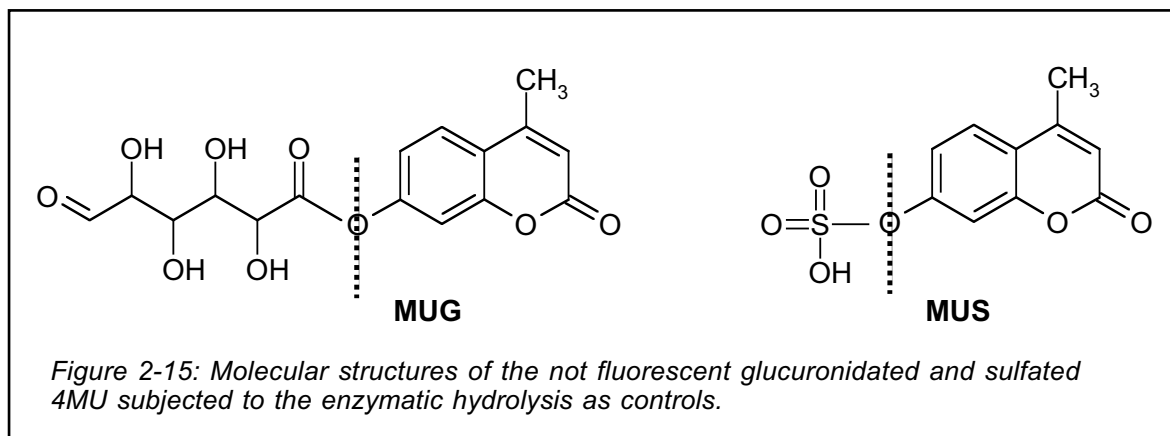


Figure 2-14:  
4-Methyl-umbelliferone,  
fluorescent

### External standards

Duplicate controls were included in each preparation:

Not fluorescent 4MU-glucuronide (4MUG) and 4MU-sulfate (4MUS) served as controls of hydrolysis (Figure 2-14). Working with equimolar amounts of 4MU, 4MUG and 4MUS, the same peak area of 4MU can only be detected, if cleavage by *Helix pomatia* enzymes is successful and complete (Figure 2-15).



4MU was reference for hydrolysis controls MUG and MUS and control of serum extraction (comparing it to internal STD, see below).

Final concentration of external STDs was  $10^{-4}$ M in serum, ethanol not exceeding 2%.

#### Internal standard

After serum extraction and ethanol elution 5 $\mu$ l of 4MU ( $10^{-2}$ M) were added to all samples (except external standards) being an internal control of reconstitution

Each sample AREA was corrected by the relative recovery of its internal STD.

#### 2.6.3.4 HPLC conditions

**Column** 250x4,6mm

#### Autosampler

Injection volume 20 $\mu$ l

Sample loss 10 $\mu$ l

**UV-detector** - runs at optimum wavelength for respective analyte

GEN 260nm

8PN 220nm

RES 305nm

4MU 320nm (external STD)

#### Fl-detector

excitation 330nm emission 374nm

Wavelengths were selected for the optimum signal of RES, which is also detectable by fluorescence (Stecher *et al.* 2001). These settings also allowed reliable and reproducible detection of internal STD 4MU. Fluorescent chromatograms were acquired with every run.

## Gradients

RES required development of a different gradient to achieve baseline separation from the int STD 4MU.

Table 2-13: Gradients and retention times of test and control substances for serum analysis

min	A %	B %	min	A %	B %
0	70	30	0	80	20
15	25	75	20	75	25
16	0	100	21	0	100
21	0	100	26	0	100
22	70	30	27	80	20
29	70	30	32	80	20

retention time	UV		FI		retention time	UV		FI	
<b>GEN</b>	9,1 min				<b>RES</b>	17,0 min		17,2 min	
<b>8PN</b>	13,1 min				<b>4MU</b>	13,1 min		13,3 min	
<b>4MU</b>	6,5 min		6,7 min						

### 2.6.3.5 Recovery of analytes from serum

Compared to EtOH dilutions the detected area after extraction from serum was reduced by  $10^6$  times for all substances. Therefore the STD-curves were obtained from serum spikes.

### 2.6.3.6 STD-curves of analyte in serum

The minimum concentration for the STD-curve in serum was set at the system sensitivity limit of the substances in EtOH (see HPLC 2.6.1 Pure compounds p.33).

The maximum concentration was derived from estimation of maximal serum levels that might be achieved and set at  $1 \cdot 10^{-3} \text{M}$ .

All STD-points were spikes of one serum-pool (final conc. of EtOH in serum 1%) and treated exactly as regular unknowns with addition of HP, incubation etc.

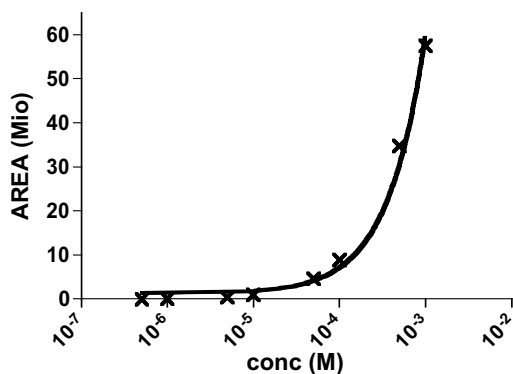
Table 2-14: Distribution of STD-points in serum

0	1	2	3	4	5	6	7	8	9	10
blank	$5 \cdot 10^{-8} \text{M}$	$1 \cdot 10^{-7} \text{M}$	$5 \cdot 10^{-7} \text{M}$	$1 \cdot 10^{-6} \text{M}$	$5 \cdot 10^{-6} \text{M}$	$1 \cdot 10^{-5} \text{M}$	$5 \cdot 10^{-5} \text{M}$	$1 \cdot 10^{-4} \text{M}$	$5 \cdot 10^{-4} \text{M}$	$1 \cdot 10^{-3} \text{M}$

For all 3 substances peak areas were still within linearity of the curve at  $5 \cdot 10^{-7} \text{M}$  in serum, while signals were not detectable any more at  $1 \cdot 10^{-7} \text{M}$ .

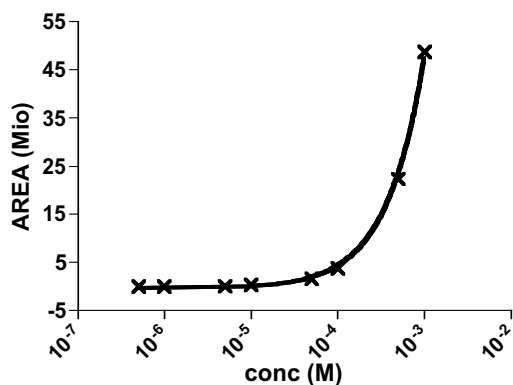
Resulting areas were plotted with log x-axis to see all STD-points, and linearity of the curve was calculated. Since the serum-pool was obtained from soy-fed rats, it was necessary to subtract the resulting background for the GEN STD-curve.

**STD-curve GEN in serum**



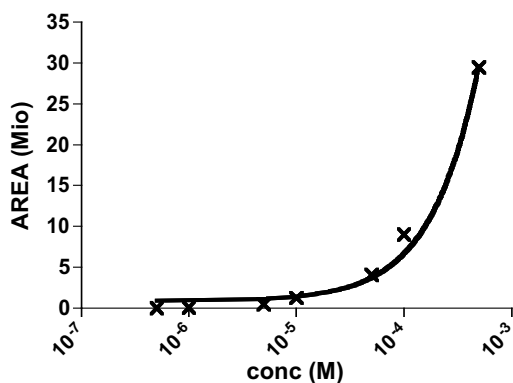
GEN	
Best-fit values	
Slope	58650 ± 2428
Y-intercept when X=0.0	1.240 ± 0.9647
X-intercept when Y=0.0	-0.00002115
1/slope	0.00001705
95% Confidence Intervals	
Slope	52710 to 64600
Goodness of Fit	
r squared	0.9898

**STD-curve RES in serum**



RES	
Best-fit values	
Slope	48490 ± 802.0
Y-intercept when X=0.0	-0.4717 ± 0.3186
X-intercept when Y=0.0	0.000009727
1/slope	0.00002062
95% Confidence Intervals	
Slope	46530 to 50460
Goodness of Fit	
r squared	0.9984

**STD-curve 8PN in serum**



8PN	
Best-fit values	
Slope	58250 ± 2057
Y-intercept when X=0.0	0.8499 ± 0.3985
X-intercept when Y=0.0	-0.00001459
1/slope	0.00001717
95% Confidence Intervals	
Slope	53760 to 62730
Goodness of Fit	
r squared	0.9853

Figure 2-16: Resulting STD curves from serum spikes in logarithmic plots to visualize distribution of STD points along the regression line.

## 2.7 Serum analysis

### 2.7.1 Hormones

Endocrine parameters were analyzed by various radioimmunoassays (RIA). Sample antigen and tracer antigen compete for binding to the primary antiserum. The antigen-primary antiserum complex is precipitated with a secondary antiserum for separation from unbound surplus tracer. The amount of bound tracer concentration is inversely proportional to hormone concentration of the sample. The pituitary hormones LH, FSH, TSH, and prolactin were measured by specific RIA established in the laboratory, for which reference preparations (standard curve), hormones (rat specific iodination preparation) and primary antiserum (anti-rat) were supplied by the National Hormone and Pituitary Program of the NIH. The respective tracer-hormone had to be  $^{125}\text{J}$  labeled before usage (Hunter *et al.* 1962). Sample volumes depended on the amount and type of hormone (*Table 2-15*), as well as the age of tracer (max. 4 weeks) and were diluted in final 500 $\mu\text{l}$  with 1% BSA (Serva 11930 albumin bovine fraction V pH 7,0) in PBS (pH 7,2).

*Table 2-15: Overview of sample volumes used ( $\mu\text{L}$ ):*

	LH	TSH	FSH	Prolactin
Cell culture	5	50		
Single dose	20	50		
Long term	25	50	100	25

Samples were first incubated with 200 $\mu\text{l}$  primary antibody (diluted with normal rabbit serum 1:300 in PBS) for 24h, after addition of 100 $\mu\text{l}$  tracer (ca. 20.000cpm) for 24h and finally with 200 $\mu\text{l}$  secondary antibody (antiserum rabbit  $\gamma$ -globulins 1:30 in PBS) for another 48-72 h. After centrifugation (60 min, 15.000g) tubes were decanted, counted for at least 1 min. Count data was evaluated by the Ria-Calc program (Wallac).

STD-curves included total counts, unspecific binding (normal rabbit serum), specific binding and 10 STD-points in triplicates covering ranges shown in *Table 2-16*:

STD-curve range	LH	TSH	FSH	Prolactin
(ng/ml)	0,005-5,12	0,01-10	0,2-64	0,1-128

Commercially available kits by DSL (Sinsheim, Germany) were used for determination of

- E2 (Estradiol 3<sup>rd</sup> Generation)
- total T3 and T4 (Active<sup>™</sup> T3/ Active<sup>™</sup> Thyroxin)
- calcitonin (DSL-1200)

Free T3 and T4 levels were assayed with RIAZENcoFT3 and RIAZENcoFT4 kits from ZenTech (Angleur, Belgium). For rat leptin a RIA kit from Linco Research was obtained. Details of the commercially available assays are described in data sheets provided by the distributor.

### **2.7.2 Metabolic parameters**

Serum triglycerides, cholesterol, LDL- and HDL-lipoproteins were determined by a Hitachi autoanalyzer (Roche) utilizing commercially available kits. N-MID osteocalcin and serum  $\beta$ -CrossLaps were measured with electrochemiluminescence immunoassays for Elecsys analyzer (Roche).

## **2.8 Real-time PCR**

Gene expression was determined by two-step real-time reverse transcription polymerase chain reaction (RT-PCR). First RNA obtained from the samples is reversely transcribed to cDNA, then PCR (Mullis *et al.* 1992) is performed. The real-time PCR system is based on the detection and quantitation of a fluorescent reporter (Livak *et al.* 1995) as an indicator of amplicon production during each PCR cycle (i.e. in “real time”) as opposed to the endpoint detection.

### **2.8.1 RNA-extraction**

Total RNA was extracted from pituitary gland, uterus, thyroid and liver, which were stored at  $-70^{\circ}\text{C}$  until RNA preparation. Uterus and liver samples were chilled in liquid nitrogen and pulverized in a 5ml-teflon container with a tissue homogenizer (Micro-dismembrator™, Braun, Melsungen, Germany). The RNeasy™ Mini Kit (Qiagen, Hilden, Germany) was used for further processing of the samples following the instructions of the manufacturer. 50mg of tissue powder was suspended in 600 $\mu\text{l}$  RT-lysis buffer and further homogenized by brief ultrasound sonication (10 sec). Of thyroid half of the organ was processed: Samples were transferred in 300 $\mu\text{l}$  of RT-lysis buffer and reduced into small pieces with Ultra-Turrax before sonication. The homogenates were applied onto QIAshredder™ columns and the eluates were subsequently loaded on the extraction columns. Pituitaries were transferred into 150 $\mu\text{l}$  RT-lysis buffer, cut to small pieces with a hypodermic needle and sonicated before directly loading them onto the extraction columns. Residual DNA was eliminated by enzymatic digestion with DNase I (kit component). Total RNA was eluted with DEPC-treated water: 50 $\mu\text{l}$  for uterus, 35 $\mu\text{l}$  for pituitary. To determine RNA concentrations, 5 $\mu\text{l}$  RNA solution was diluted with 75 $\mu\text{l}$  water. Absorption was measured at 260 and 280 nm with a photometer (biophotometer, Eppendorf, Hamburg, Germany). Concentrations of

the RNA solutions were adjusted to 10ng RNA/ $\mu$ l (uterus, thyroid) or 20ng RNA/ $\mu$ l (pituitary, liver) with DEPC-treated water and stored at -70°C until further analysis.

### 2.8.2 Reverse transcription

The RT-reaction was carried out with either 100ng total RNA (uterus, thyroid) or 200ng total RNA (pituitary, liver):

10 $\mu$ l RNA

1 $\mu$ l random primer 100ng

3 $\mu$ l DEPC-treated water

The mixture was incubated at 70°C for 10 min to disrupt secondary structures.

Then the following reagents were added:

4 $\mu$ l 5x reaction buffer (250mM Tris-HCl, 375mM KCl, 15mM MgCl<sub>2</sub>, 50mM dithiothreitol)

1 $\mu$ l deoxy-NTP mix PCR grade 10mM

1 $\mu$ l M-MLV reverse transcriptase RNase H<sup>-</sup> 200U

0,1 $\mu$ l recombinant RNasin ribonuclease inhibitor 4U

---

20 $\mu$ l total volume

Reverse transcription was performed in a thermocycler:

10 min at 22°C starting incubation

50 min at 42°C enzymatic reaction

10 min at 95°C inactivation of the enzyme, denaturing of RNA-cDNA hybrids

All cDNA-samples were stored at -20°C.

Reagents except random primer (Gibco, Karlsruhe, Germany) and dNTPs (Invitrogen) were purchased from Promega (Mannheim, Germany).

### 2.8.3 Taqman®-PCR-Assay

Real-time PCR reactions were based on the 5' nuclease assay (Heid *et al.* 1996).

Fluorogenic probes are used, that carry a fluorescent reporter (6-carboxy-fluorescein, FAM) at the 5'-end and quencher at the 3'-end (6-carboxy-tetramethyl-rhodamine, TAMRA). First primers and probe hybridize with single strand cDNA. Due to its 5'-3'-exonuclease activity the AmpliTaq DNA polymerase hydrolyses the probe during the extension phase. The fluorescence resonance energy transfer (FRET) between reporter and quencher is cut and fluorescence of the reporter can be detected. The taq-DNA-polymerase is able to tolerate temperatures of up to 95°C briefly. By repeating the cycle of DNA-denaturation, hybridization and polymerase reaction number of generated amplicons and resulting signal grow proportionally.

**Table 2-17: Sequences of primers and probes analyzed.** (FAM 6-carboxy-fluorescein, TAMRA 6-carboxy-tetramethyl-rhodamine)

Gene	Primers: Forward Reverse	Probe (5'-FAM – 3'TAMRA)	Product size	Reference (Accession # )
ER $\alpha$	5'-AAGCTGGCCTGACTCTGCAG-3' 5'-GCAGGTCATAGAGAGGCACGA-3'	5'-CGTCTGGCCCAGCTCCTCCTCATC-3'	144 bp	(Spreafico <i>et al.</i> 1992) X61098
ER $\beta$	5'-GAGGAGATACTACTCTTCGCAATC-3 5'-GGAGTATCTCTGTGTGAAGGCCAT-3	5'- CAGGGCATCTGTCCACCGCGTTCAG-3'	159 bp	(Kuiper <i>et al.</i> 1996) U57439
FR	5'-CTGGTTCCGCCACTCATCAA-3' 5'-TCAGGCTCATCCAGGAGTACTGA-3'	5'-CCGACACTTCCAGCTCTTTGCTGACCA-3'	229 bp	(Park-Sarge <i>et al.</i> 1994) L16922
IGF1	5'-TGTCGTCTTCACATCTCTTCTACCTG-3' 5'-CCACACACGAACTGAAGAGCGT-3'	5'-TTACCAGCTCGGCCACAGCCGGAC-3'	121 bp	(Roberts <i>et al.</i> 1987) M15481
C3	5'-CTGTACGGCATAGGGATATCACG-3' 5'-ATGCTGGCCTGACCTTCAAGA-3'	5'-ATGCCATCCTCACAACACTTCCGCAG-3'	199 bp	(Misumi <i>et al.</i> 1990) X52477
$\alpha$ subunit	5'-TCTTGGACCTTGCGGGAGT-3' 5'-GGTGCCCCCATCTATCAGTG-3'	5'-TGCCCTGGAGAAGCAACAGCCCAT-3'	70 bp	(Godine <i>et al.</i> 1982) V01252
LH $\beta$	5'-ACCTTACCACCAGCATCTGT-3' 5'-AGCTCACGGTAGGTGCACACT-3'	5'-CTGCCTTGCCCTCCCGTGCCTCA-3'	104 bp	(Chin <i>et al.</i> 1983) NM 012858
TSH $\beta$	5'-GATGTACGTGGACAGGAGAGAGTGT-3' 5'-GACATCCTGAGAGAGTGCCTACTTG-3'	5'-TCAACACCACCATCTGCGCTGGG-3'	130 bp	(Strausberg <i>et al.</i> 2002) BC 058488



All systems used were validated procedures of the laboratory and are mostly published (Roth *et al.* 2001; Seidlova-Wuttke *et al.* 2003a). Primers and probes were chosen with the assistance of the Primer Express software (PE Applied Biosystems, Weiterstadt).

Oligonucleotides were purchased from Eurogentec (Seraing, Belgium). The sequences of primers and probes for the genes analyzed in this work are summarized in *table 2-17*.

Each PCR run included min. 6 duplicate cDNA samples of defined concentrations to generate a standard curve, no template controls to check for contaminations and the respective sample cDNAs.

Reagents pipetted into Optical 96-well reaction plates and covered with optical caps (MicroAmp®, PE Applied Biosystems) for measuring the fluorescence emission in the ABI Prism 7700SDS.

Amplification reactions (24µl) contained:

1X TaqMan™ Universal PCR Master Mix (PE Applied Biosystems)

50-900 nM of each primer (for details see *table 2-6*)

225 nM probe

2 - 4 µl cDNA

Total volume was adjusted with Ampuwa water.

*Table 2-18: Genes and required primer amounts*

nM	ER $\alpha$	ER $\beta$	$\alpha$ subunit	LH $\beta$	TSH $\beta$	IGF1	C3	PR
sense	50	300	300	300	300	300	900	300
antisense	900	300	300	300	300	300	900	300

Real-time PCR was run on an ABI Prism 7700 Sequence Detection System (TaqMan™, PE Applied Biosystems) under the following cycling conditions:

#### **Initial Step**

2 min at 50°C                    elimination of carryover PCR products by uracil DNA glycosylase  
10 min at 95°C                    activation of the AmpliTaq Gold DNA polymerase (T optimum 70°C)

#### **40 cycles**

15 s at 95°C                    denaturation of double strands  
1 min at 60°C                    hybridization, extension (release of fluorescent reporter)

Standard curves:

To generate cRNA, each PCR product was cloned into the pCR™ II-TOPO plasmid using the TOPO TA Cloning™ Kit following the manufacturers instructions (Invitrogen, Groningen, The Netherlands). After confirming the sequence and orientation of the cloned PCR product by

commercial sequencing (Seqlab, Göttingen, Germany), the plasmid construct was linearized with an appropriate restriction endonuclease and purified using the Wizard™ DNA Clean-Up System (Promega, Mannheim, Germany). RNA was synthesized according to the instructions of the manufacturer using the RiboMAX™ Large Scale RNA Production System with either SP6 - or T7 - polymerase, respectively (Promega, Mannheim, Germany). After photometric determination of concentrations of the RNA stock solutions, serial dilutions (10 fold intervals) of the RNA were reverse transcribed as described above. The standard curve was generated by plotting the known cDNA concentrations versus the corresponding threshold cycle ( $C_t$ ) value obtained in the real time PCR reaction using Sequence Detection Software (PE Applied Biosystems). To determine the relative expression levels of the tissue samples, the respective  $C_t$  values were interpolated from the standard curve.

## 2.9 Histology

### 2.9.1 Sample preparation

One uterine horn and vaginae were placed in 10% formalin solution immediately after removal and fixed for 48h. Then uteri were cut into three pieces of the proximal, medial and distal part of the horn. The thyroids were stored at  $-70^\circ$ , parted in halves still frozen, one half allowed to thaw in 10% formalin being fixed there for 24h.

Uterus and thyroid were dehydratized with a histokinnet (TP 1020, Leica), while vaginae were processed manually. Afterwards they were embedded with a paraffin dispenser (EG 1060, Leica). Transversal sections of 3  $\mu$ m were cut with a RM 2135 microtome (Leica) and placed on slides with permanent positive charge on the surface (Superfrost plus), which improves binding between formalin fixed sections and the glass.

### 2.9.2 Staining techniques

#### Hematoxylin and Eosin (HE)

All tissues were treated according to standard HE protocol and mounted.

Hematoxylin (Haematoxylin Mayer, Merck) dilutions were pretested for staining strength.

Eosin yellow (Merck) was used in a concentration of 0,25% in EtOH 80%.

#### Periodic acid Schiff (PAS)

Thyroid cuts were stained with HE and the PAS method. For PAS slides were immersed into 1% solution of periodic acid for 10min, followed with periodic acid Schiff (Lillie's cold Schiff's reagent) for 30min at RT and counterstained with hematoxylin.

**Papanicolaou**

Vaginal smears were immediately fixed in isopropanol for 20 min. The Papanicolau staining (Romeis 1989) of the slides was performed with an automated stainer at the Dept. of Gynecology and Obstetrics, University Clinic, Göttingen.

**2.9.3 Analysis**

Tissues were analyzed with a Axiophot microscope (Zeiss). Microscopic pictures were taken with a Color View 12 camera (SIS) and transferred to the computer by the AnalySIS image processing program (SIS). Histological sections were inspected groupwise and blinded to reduce bias and ensure correct classification.

**2.9.3.1 Uterus**

Estradiol induces morphological changes in uterus at both physiological and supraphysiological serum levels. These changes are described in literature (Haschek *et al.* 2001) and allow comparison to those of any test substances.

The analyzed features are displayed in *Figure 2-17* on the next page.

**Physiological serum levels of E2**

Generally all these findings can be detected along an estrus cycle. Nuclei of the lamina propria stromal cells are round when lacking E2 stimulation (A2) and adopt a spindle shape and an organized pattern (polarity) at physiological high levels (B2).

E2 induces a renewal of epithelial cells (endometrial and gland epithelium, A1+3), therefore necrosis (or apoptosis) and mitosis are exerted. The epithelial cells also tend to show a high cytoplasmic to nuclear ratio (secretory function) (B1+3), while appearing cuboidal with a small cytoplasmic/nuclear ratio when lacking of E2 stimulation (A1).

**Supraphysiological serum levels of E2**

Additional findings appear, which are considered pathological. Overdosed stimulation causes further proliferation and secretion. Characteristics evaluated are hypertrophy and hyperplasia of epithelial glands (D3) and multilayered endometrium epithelium (C1), which might develop squamous metaplasia (C arrow). Since E2 also opens cervix, its constantly high level increases the risk of developing pyometra.

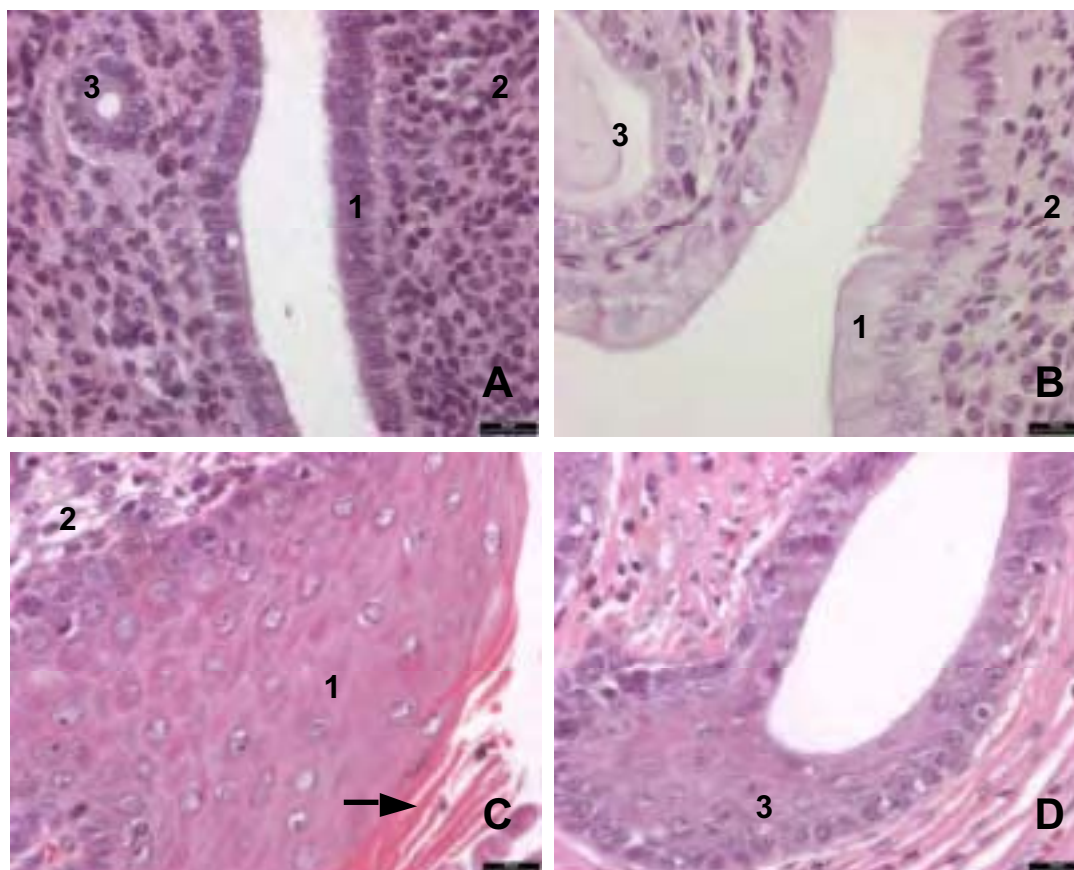


Figure 2-17: Transversal sections of uterus, original pictures taken with 20x magnification

- (1) epithelial cells along the lumen
- (2) lamina propria cells in the stromal layer
- (3) endometrial gland with lining epithelium

Uterus ovx:

**A** single layered endometrium and gland epithelium with low cytoplasmic/nuclear ratio, loose organisation of round lamina propria cells

Physiological E2 levels (intact animal):

**B** single layered endometrium and gland epithelium with high cytoplasmic/nuclear ratio, polarized organisation of spindle shaped lamina propria cells

Supraphysiological E2 levels (ovx, E2 treated):

**C** multilayered epithelium with squamous metaplasia

**D** hyperplastic gland with hypertrophic epithelial lining

### 2.9.3.2 Vagina

In vagina E2 influence mainly affects epithelium layers, increasing their number and inducing cornification. Along an estrus cycle the number of layers normally varies between 5 and 9, and cornification only appears during estrus after maximum exposure to E2 (Haschek *et al.* 2001). In vaginal smears the appearance of large polygonal cornified

epithelial cells is the main feature of E2 influence. With Papanicolaou stain (PAP) cornified cells are orange in contrast to the blue-green small basal cells. This polychromatic technique allows detecting intermediate stages that might be induced by test substances. The condition of nuclei is a further criterion.

### **2.9.3.3 Thyroid**

In the normal thyroid follicles are lined by a simple cuboidal epithelium responsible for thyroglobulin and T3/T4 production. C-cells are named “clear” for lesser staining and are also large in comparison to the follicular cells. They secrete calcitonin and are located among the thyroid follicular cells and near the capillaries, without contact colloid.

([www.kumc.edu/instruction/medicine/anatomy/histoweb/index.htm](http://www.kumc.edu/instruction/medicine/anatomy/histoweb/index.htm))

Characteristics for disrupting effects were defined according to a histopathological assessment in a thyroid screening battery (O'Connor *et al.* 1999).

Primary change found is colloid depletion. Thyroglobulin in the colloids can be visualized with PAS stain, which produces a deep red appearance of carbohydrates.

Also number, shape and diameter of follicles are possibly affected. Hypertrophy and hyperplasia of the whole gland may be found due to an increased number of follicular cells.

## **2.10 Computer tomography**

Bone mineral density was determined to record ovariectomy induced osteoporosis in rats (Helterbrand *et al.* 1997). (see Long term treatment 2.5.2 Measurements during treatment period, p.31)

### **2.10.1 Instrument**

XCT Research SA+ (Stratec Medizintechnik, Pforzheim, Germany):

Determination of bone density and geometrical parameters of the bone by peripheral quantitative computer tomography (pQCT) for research purposes

### **2.10.2 Procedure**

Animals were anaesthetized with isoflurane (Forene®, Abbott) and placed in the tomograph. After a scoutview scan along the leg a reference line is placed through the most proximal site of the joint. This is necessary to enable exact positioning of repeated measurements.

The actual measurement level through the metaphysis of the tibia lies in defined distance from the reference line down the bone.

The transversal picture is obtained by 15 linear scans across the bone axis, that move around the leg and cover 180°. Additional scans through metaphysis and diaphysis lie in 0,5mm and 1,5mm distance from the first section.

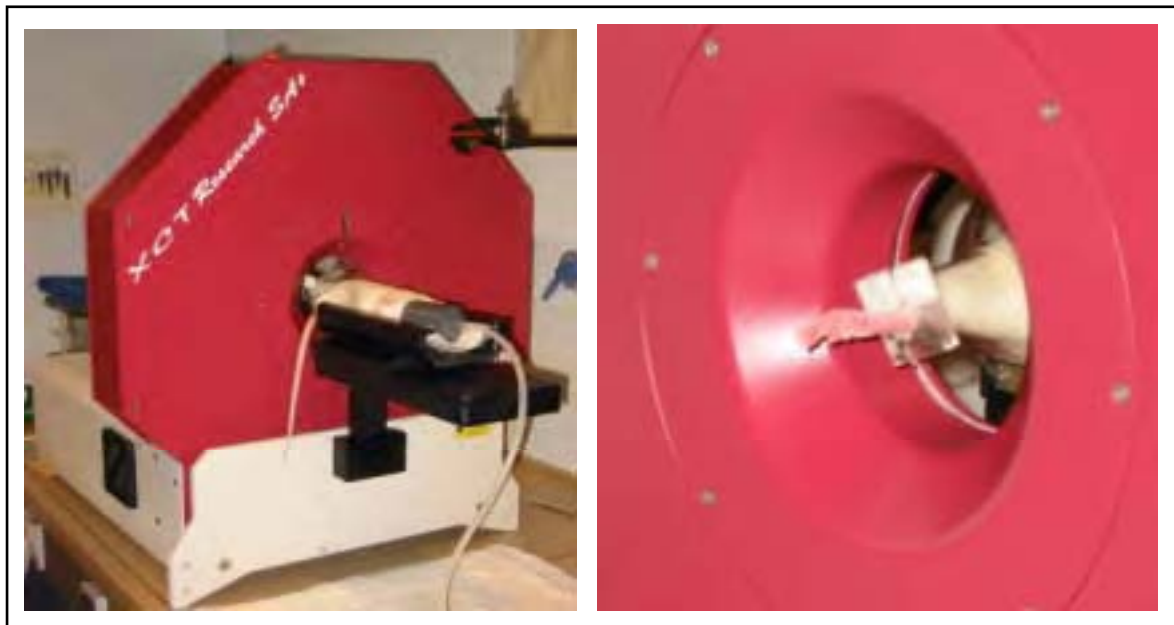


Figure 2-18:  
Position of the animal and its leg in the computer tomograph for the tibia scan.

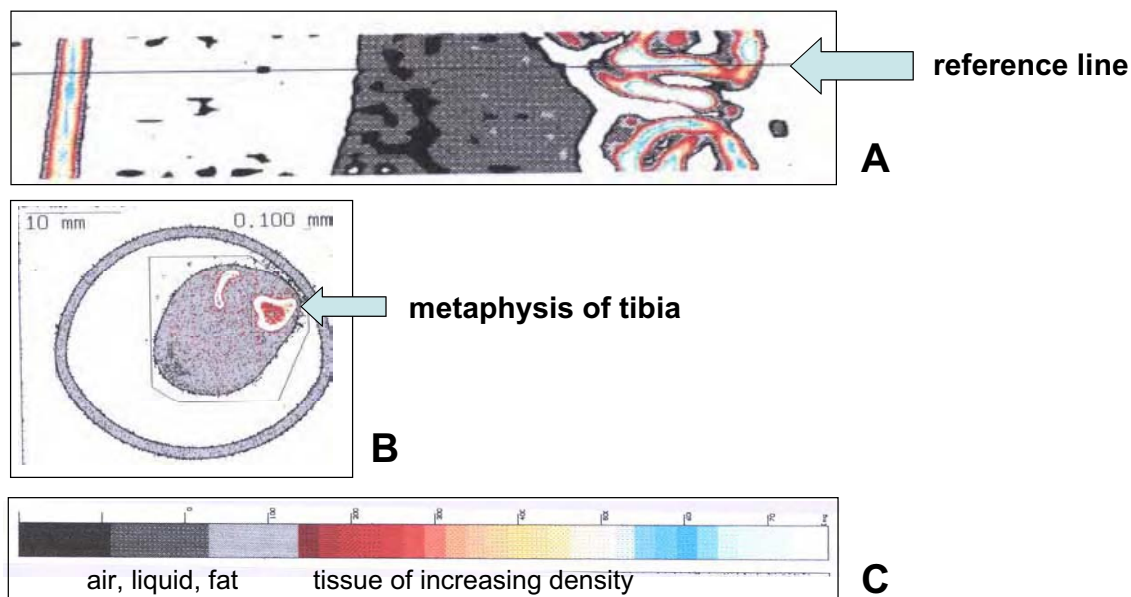


Figure 2-19:  
**A)** Tomographic picture displayed after the scoutview scan and illustration where the reference line was placed.  
**B)** Example of the final transversal scan for density evaluation.  
**C)** Color code of the tomograph to discern varying density.

### 2.10.3 Evaluation

Each CT-measurement was evaluated by manually marking the region of interest, i.e. the metaphysis of the tibia. Differentiation of tissues by density was preferred to purely geometrical or mixed methods. First bone and soft tissue are separated by a threshold algorithm ( $280\text{mg}/\text{cm}^3$ ). Then an inner threshold ( $400\text{mg}/\text{cm}^3$ ) divides spongiosa and corticalis.

This procedure discerned trabecular and cortical density ( $\text{mg}/\text{cm}^3$ ) yielded and mass (mg) values. The software also calculated the “polar strength strain index” SSI ( $\text{mm}^3$ ), which represents stability of the bone regarding bending and torsion along the bone axis. It is derived from correlation of geometrical properties with the determined cortical density. From basal CT of all animals before treatment the mean was calculated and set as reference value. The individual values of final CT after 3 months treatment were %-related to the reference value and the resulting means of control and treatment groups compared statistically.

## 2.11 Data compilation and statistical analysis

The statistical re-evaluation of several endocrine disruptors low-dose studies supported by The National Institute of Environmental Health Sciences (NIEHS) and the U.S. Environmental Protection Agency (U.S. EPA) led to publication of guidelines regarding appropriate experimental design and statistical analysis of experimental studies (Haseman *et al.* 2001) (complete report is available on the National Toxicology Program web site <http://ntpserver.niehs.nih.gov/>). It was tried to include as many aspects of their recommendations as possible for design, evaluation and interpretation of this work.

### Study design

Sensitivity of the study depends on sample size, the underlying variability of the data, the magnitude of the treatment effect that is present, the method of statistical analysis and the level of significance. The main potential of study design lies in reducing variability of response.

*Replication* within an experiment was considered, as well as potential *bias*.

For *quality control* written record was kept, raw data handled with utmost care and only validated methods applied for measurement of variables, also ensuring *reproducibility*.

### **Data Analysis Issues**

Prior to any statistical analysis individual data was visually inspected for plausibility to identify outliers. Also any measurement was tabulated for each animal to detect individual sensitivity to treatment. Mean, standard deviation (SD) and standard error (SEM) of the mean were calculated for all data. Graphs show mean $\pm$ SEM, if not stated otherwise. All statistical procedures were utilizing Prism™ software (Graph Pad, San Diego, USA).

#### *Choice of statistical methodology*

One-way analysis of variance (ANOVA) was chosen as primary statistical method, followed by a post-hoc test for multiple comparison (Dunnett) without requiring statistical significance of the overall ANOVA. Distribution within groups was tested by the D'Agostino and Pearson test. If distributions were not normal for and variances heterogeneous (Bartlett's test) for most groups, data was transformed logarithmically before reanalysis by ANOVA. If transformation was unsuccessful, data was evaluated with Kruskal-Wallis test followed by Dunn's test, since the loss of power of a nonparametric test on normal distribution is less than risk of false-negative outcomes ignoring not normal distribution (Glantz 2002).

Values of  $p < 0.05$  were considered to be statistically significant.

In the present study *relative* changes of mRNA levels were analyzed in comparison between the control and treatment groups. Based on the  $C_t$  values of the PCR reactions measured in the control group, an average expression level of the analyzed target gene was calculated from the standard curve, which was set 100 percent. Individual  $C_t$  values in the treatment groups were transformed in relation to this average value of the control group.

#### *Adjusting organ weight for body weight*

Organ weights and organ/bw ratios are presented, since some organs are independent of body weight and others are not, though for metabolic organs relative weights are often preferred. The recommended analysis with bw as covariable is not applicable because the covariable should be independent of treatment, while E2 is known to affect organ and body weight.

#### *Biological interpretation*

When interpreting statistically significant effects it was considered, whether they are of biological importance (positive/adverse). Respectively attention was paid to important end points that might be affected though not exhibiting statistical significance.



### 3 Results

#### 3.1 Pituitary cell culture

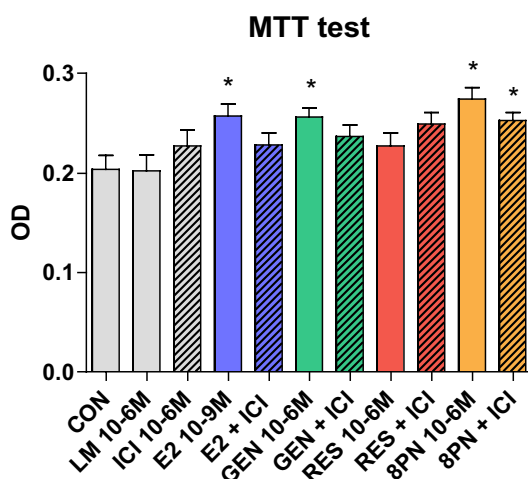
The cell culture experiments were supposed to provide information of potential impact of the test substances on HPG- and HPT-axes at the pituitary level and was performed in duplicate.

In all concerned graphs statistical significances are marked the following:

- \*  $p < 0,05$  vs. CON
- °  $p < 0,05$  compound coincubated with ICI vs. pure compound

##### 3.1.1 Viability

After 48h of incubation with the test substances no toxic effects were found. In the MTT test cells were equally viable as control or enhanced viability detectable under E2, GEN and 8PN treatment (*Figure 3-1*). The reported interference of phytoestrogens with the MTT (Bruggisser *et al.* 2002), producing a blue staining without cells present, was tested and not confirmed in this system.



*Figure 3-1: Viability test of pituitary cell cultures obtained in two experiments.*

##### 3.1.2 Effects on LH release

E2 and 8PN obviously had a strong influence on LH release by the pituitary cells. The effect of GEN and RES is not as clear, also if ICI by itself had a negative effect. All compounds except RES were significantly antagonized with ICI (*Figure 3-2*).

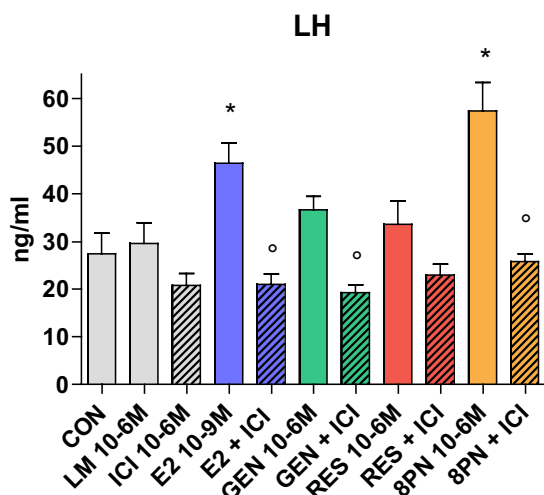


Figure 3-2: LH release by cultured pituitary cells after 48h incubation with test compounds and 3h of GnRH stimulation.

### 3.1.3 Effects on TSH release

Again results were clear for E2 and 8PN causing an increase of TSH production (Figure 3-3). For GEN the increase was not evident. Incubation with ICI significantly suppressed TSH release under E2 and 8PN treatment. GEN and RES did not show conclusive effects.

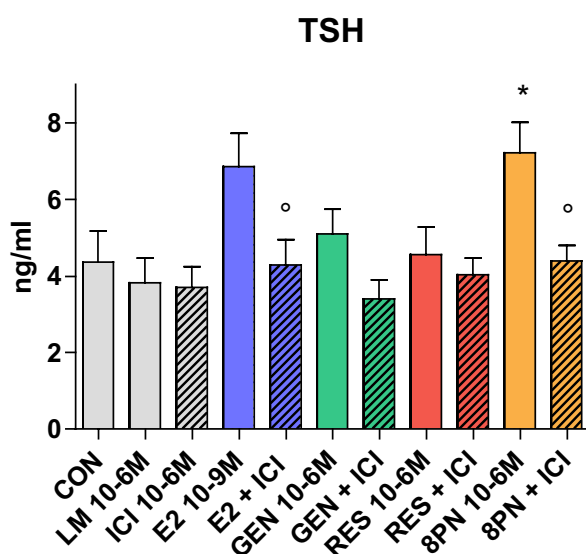


Figure 3-3: TSH release of cultured pituitary cells after 48h incubation with test compounds and 3h of TRH stimulation.

## 3.2 Single dose treatment

### 3.2.1 Serum levels

In E2 treated animals the serum levels of E2 reached effective  $324 \pm 3,5$  (SEM) pg/ml (approx. 1,2 nM). Serum levels of the three test compounds were determined by HPLC-UV. Administration of 150mg/kg bw was intended as strong dosing to provoke measurable effects within the short treatment time. Though the same amount of substances was given, total serum levels differed remarkably (Figure 3-4 A).

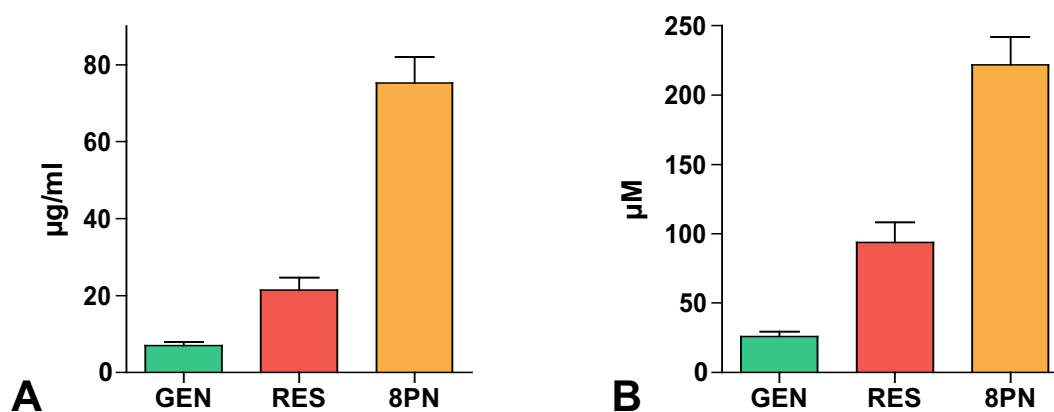


Figure 3-4: Total serum levels of test compounds after oral administration of 50mg/animal each. Concentrations are also shown in  $\mu\text{M}$  (B) to enable comparison with the equimolar administration of the long term treatment (see chapter 3.3.3.2 Effective serum levels).

### 3.2.2 HPG-axis and uterus

#### 3.2.2.1 Uterus weight

All animals entered the experiment with similar body weights (Figure 3-5). After a brief exposure time of only 6h differences in uterus weight could be detected between the groups.

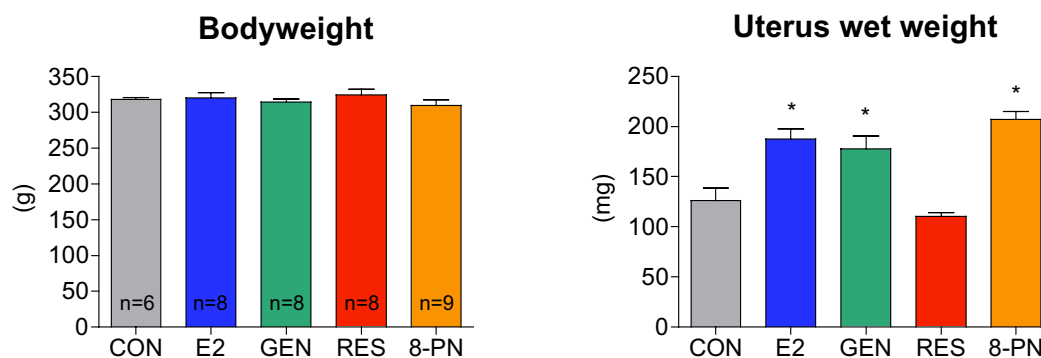


Figure 3-5: Comparison of body and uterus weight after 6h treatment time (\*  $p < 0,05$  vs. CON).

Besides E2, GEN and 8PN resulted in organ wet weight elevated by one third and more, which is likely to be due to irrigation of the tissue. RES rather shows a tendency towards decline of uterus weight.

### 3.2.2.2 LH levels in serum

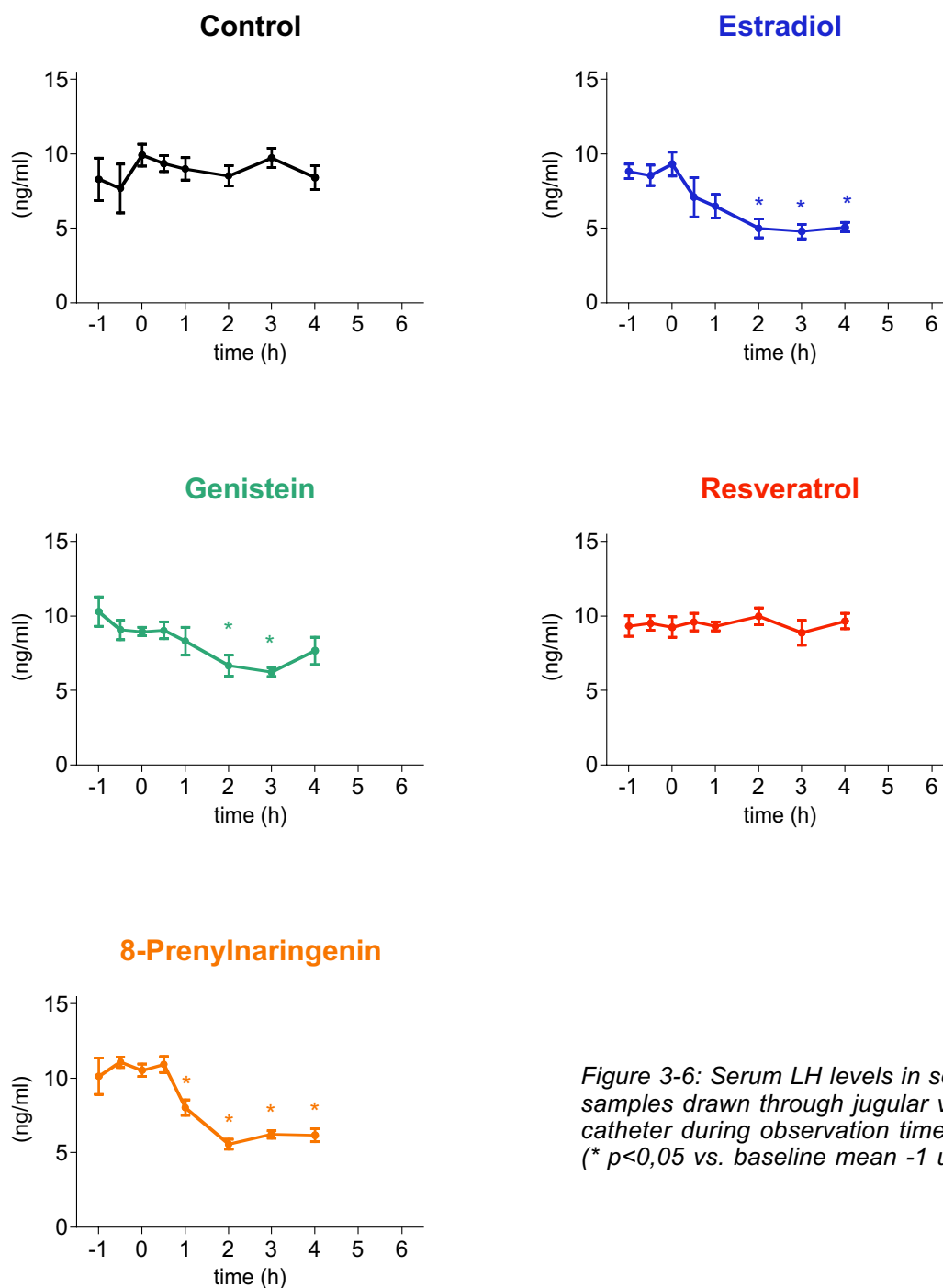


Figure 3-6: Serum LH levels in serial samples drawn through jugular vein catheter during observation time (\*  $p < 0,05$  vs. baseline mean -1 until 0h).

Samples of treatment time were compared to the baseline mean (time points -1h, 0,5h, 0h). Inspecting the development of LH levels in the serial samples over the time course, the same estrogenic activity pattern appeared as already observed for uterine weight stimulation. 8PN and less pronounced GEN suppressed LH like E2, while RES remained without response. The effect was significant for 8PN after 1h, for E2 and GEN after 2h (Figure 3-6). Under GEN treatment suppression slackened after 4h, while under the other two it persisted until the end at 6h (Figure 3-7).

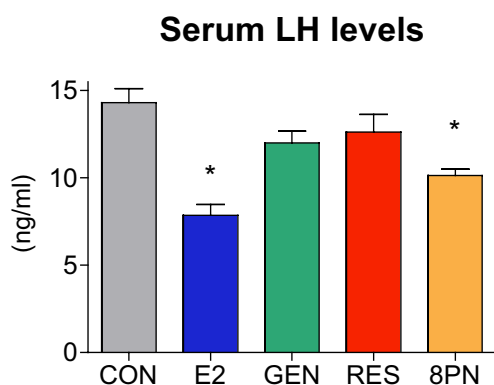


Figure 3-7:  
Serum LH levels in decapitation blood 6h after administration of the test compounds (\*  $p < 0,05$  vs. CON).

### 3.2.3 HPT-axis

#### 3.2.3.1 TSH levels in serum

E2 elevated TSH levels the most, followed by 8PN and GEN (Figure 3-9). The peak level for all three was achieved at 3 h. Serum level of GEN seemed to decline faster, since effect on TSH (as on LH, see above) faded at 4h and could not be detected in decapitation blood (Figure 3-8). RES was also ineffective concerning TSH release.

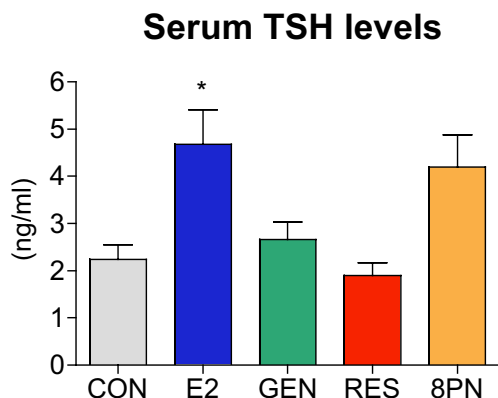


Figure 3-8:  
Serum TSH levels in decapitation blood 6h after administration of the test compounds (\*  $p < 0,05$  vs. CON).

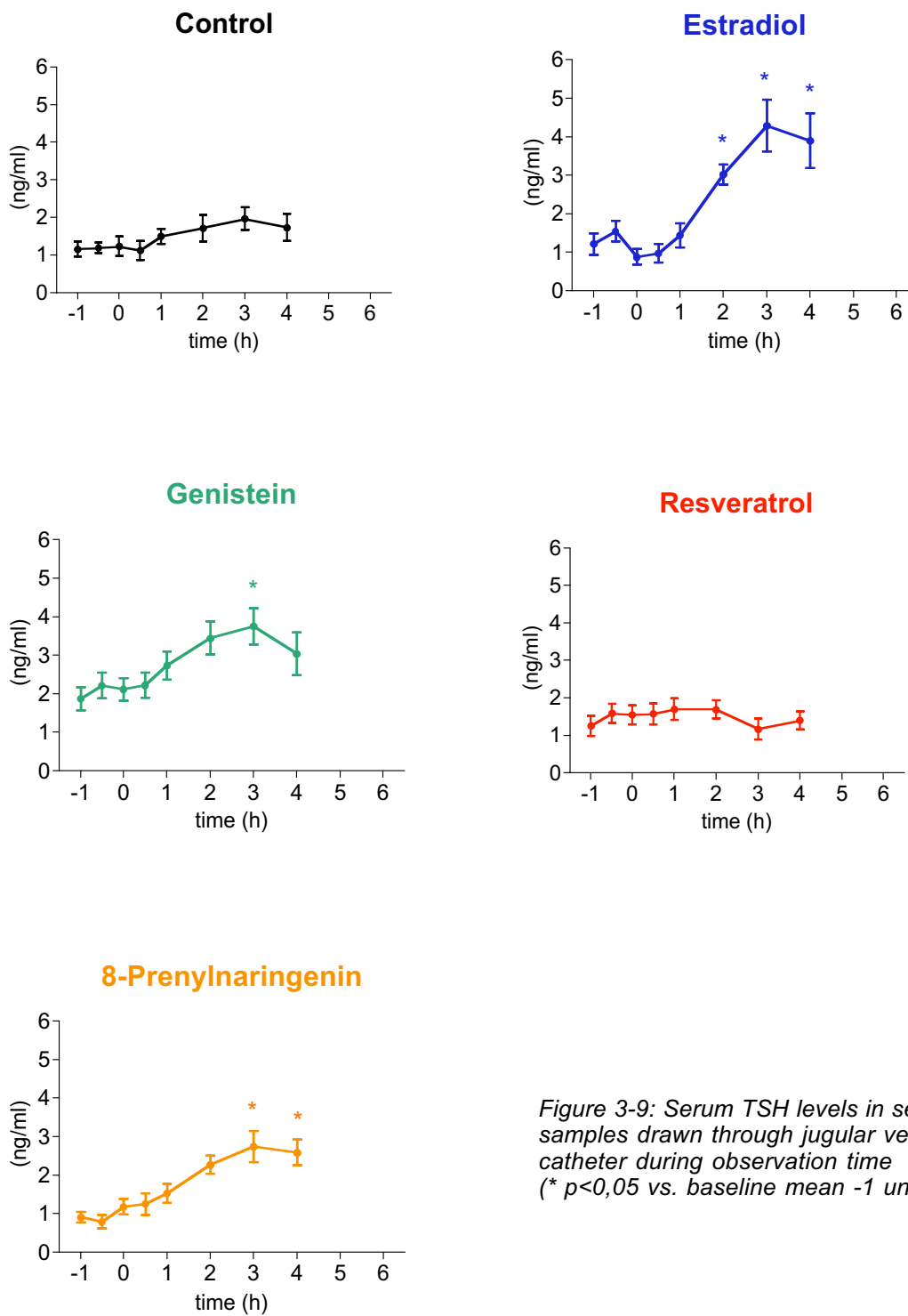
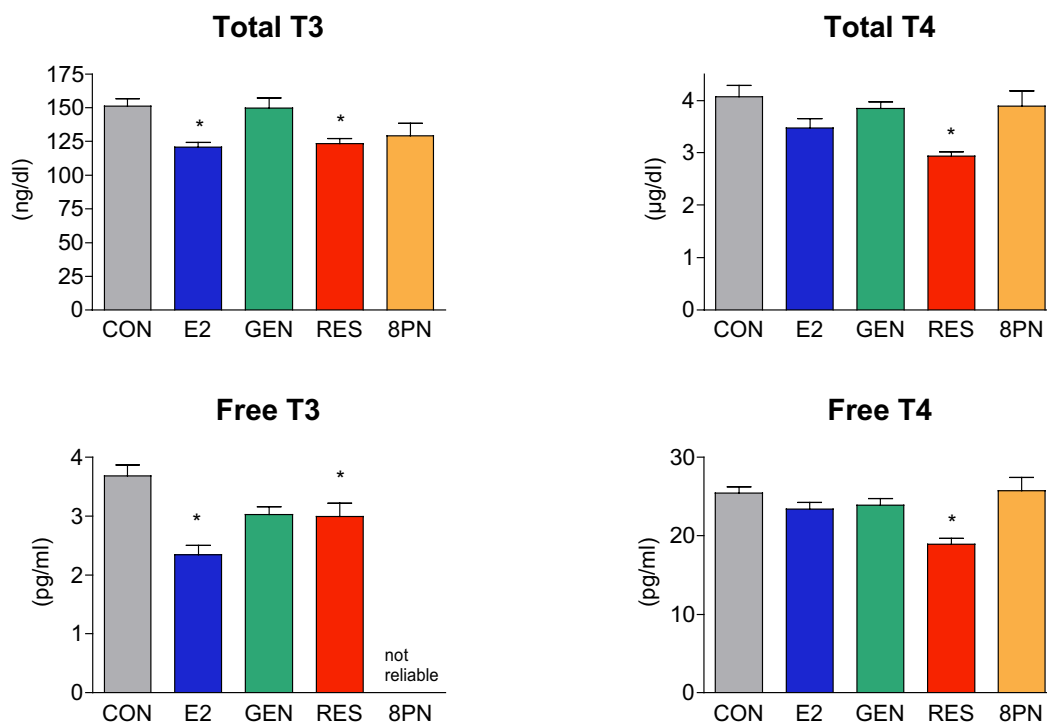


Figure 3-9: Serum TSH levels in serial samples drawn through jugular vein catheter during observation time (\* p<0,05 vs. baseline mean -1 until 0h).

### 3.2.3.2 Thyroid hormones in serum

Levels of free and bound T3 (FT3 and TT3) are shown in *Figure 3-10*. They were reduced by E2. RES had a lowering effect on free and total fractions of both thyroid hormones. 8PN showed a declining tendency for TT3, but could not be measured in FT3 assay because of an interaction of 8PN with the tracer.



*Figure 3-10:* Serum levels of bound and free thyroid hormones in decapitation blood (\*  $p < 0,05$  vs. CON).

The relations of free and total fractions (*Table 3-1*) match data from literature quite well:

- regular fraction of free thyroid hormone is less than 0,1% (Palha 2002)
- T4 release by the thyroid is more than 85% of total thyroid hormones (Hesch 1989)
- relation bound T3 to T4 in plasma is approx. 1:100 (Silbernagl *et al.* 1991).

The main alteration by treatment was caused by E2, which significantly decreased the ratio of free T3/free T4.

*Table 3-1:* Proportions of free and total fractions and among both thyroid hormones (\*  $p < 0,05$ ).

relation %		CON	E2	GEN	RES	8PN
free/total	T3	0,24	0,20	0,21	0,25	
	T4	0,06	0,07	0,06	0,07	0,07
T3/T4	free	14,5	9,7*	12,7	14,0	
	total	3,8	3,6	3,9	4,3	3,4

### 3.3 Long term treatment for 3 months

#### 3.3.1 Analysis of animal diet

To determine the accuracy of dosage with the supplemented food, HPLC-based analyses were performed.

##### 3.3.1.1 Recovery

Since the test compounds were administered as pelleted food, their recovery from powdered food was tested in spike experiments (*Figure 3-11 and Table 3-2*).

In the low concentrations the variability between spikes was generally higher. While GEN and 8PN lay in acceptable ranges, the low recovery rate for RES couldn't be improved with different extraction methods.

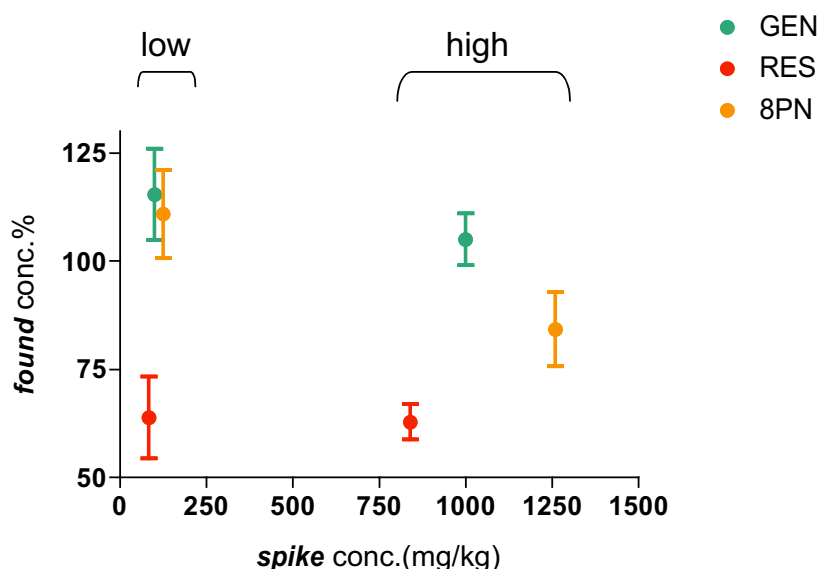


Figure 3-11: Recovery of test substances from pulverized and spiked food pellets in low and high doses (means±SD).

Table 3-2: Overview of recovery measurements in the spike experiments.

conc (mg/kg)	required	found	SD	recovery %	inter-assay	intra-assay
					SD %	mean var. %
GEN	low	100	115,42	12,30	115,4	0,51
	high	1000	1050,03	61,96	105,0	0,10
RES	low	84	53,57	5,15	63,8	0,49
	high	840	527,64	19,85	62,8	0,26
8PN	low	126	139,65	15,01	110,8	0,35
	high	1260	1060,85	89,78	84,2	0,26



The reliability of the spikes remained unclear: An exact estimation would require more repeated experiments than the two triplicates (minimum possible) executed. Also, more important, the spiking conditions were not optimal, since pellets were so hard that they were not completely pulverized before adding pure compound for the spike and crumbs did not desintegrate during ethanol-extraction. This is likely to result in higher recovery rates than achievable with already supplemented food.

### 3.3.1.2 Content

In the first week of treatment contents of the supplemented food were measured, that was manufactured the week before. The following *Table 3-3* provides an overview of ordered content of the test compounds (mg per kg of food) and the determined absolute and relative values obtained by HPLC, also comparing them to the recovery rates of the spike experiments.

8PN is quite consistent and close to expected values, while lower concentrations were found for RES and even less for GEN. Besides only relative values of recovery rates, a reduction of content may be due to the pelleting process, where pellets are exposed to temperatures of 60-70°C for up to 1 min. Instability of GEN under high temperatures has been demonstrated (Xu *et al.* 2002), while RES is sensible to temperature and light (Wang *et al.* 2002).

*Table 3-3: Comparison of content in supplemented food with expected values and recovery rates from spike experiments.*

CONTENT		required	found	± SD	%	± SD%	recovery %	SD %
conc. (mg/kg)								
GEN	low	100	81	2,5	81	3,0	115,4	10,5
	high	1000	821	5,8	821	0,7	105,0	6,0
RES	low	84	41	1,7	48	4,3	63,8	9,5
	high	840	499	25,6	59	5,1	62,8	4,0
8PN	low	126	157	1,3	125	0,8	110,8	10,2
	high	1260	1055	17,4	84	1,7	84,2	8,5

### 3.3.1.3 Stability

The first bag of pellets normally lasted for 7 weeks, but for 10 weeks in the groups GEN high and 8PN high with reduced consumption.

The substances displayed variable stability in the supplemented food as can be seen in *Table 3-4*, also differing between the 1<sup>st</sup> and the 2<sup>nd</sup> bag, which was kept at -20°C until use. GEN high and 8PN high showed stable contents, while contents of their low doses partially seemed to decline with time. For RES a more or less pronounced decrease had to be noted in any dose, which was likely due to its temperature sensitivity.

Table 3-4: Content variation in supplemented food within approx. 7 weeks of open storage.

STABILITY		1st bag				2nd bag			
conc. (mg/kg food)		start	$\pm$ SD	end	$\pm$ SD	start	$\pm$ SD	end	$\pm$ SD
GEN	low	81	2,5	76	2,5	75	1,3	72	2,9
	high	821	5,8	856	12,3	800	23,7	806	10,4
RES	low	41	1,7	31	2,3	40	1,3	37	2,7
	high	499	25,6	438	11,6	508	23,2	405	27,3
8PN	low	157	1,3	113	2,8	126	4,5	110	3,7
	high	1055	17,4	1083	15,5	1081	9,6	1094	25,4
		week 1				week 14			

### 3.3.2 Parameters during treatment

#### 3.3.2.1 Food and water intake

Daily food and water intake were monitored by measuring consumption by cage twice and three times a week, respectively. Therefore the calculated intake rates are not means of consumption attributed to individual animals, but averages of two group with 6 animals each (Figure 3-12).

Food intake stabilized within 3 weeks after ovx and start of treatment. The decline in appetite at the end might have been due to a urodynamic measurements under isoflurane anaesthesia after 11 weeks. Water intake turned out more variable, since any additional handling of cage and/or bottle resulted in variation from regular water loss.

Statistical comparison among the groups and calculation of intake and resulting mean dosage throughout the experimental time were based on the constant values between week 4 and 11.

The dot plot graph provided an overview of patterns among the groups. RES increased water consumption without effect on food, maybe due to kidney effects (Crowell *et al.* 2004). For GEN, 8PN and E2B there was a dose dependent parallel decline of food and water intake, which was differing significantly from CON.

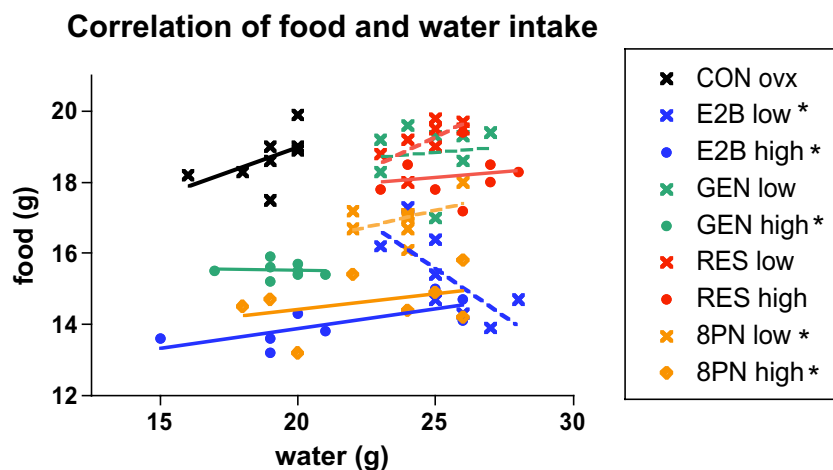
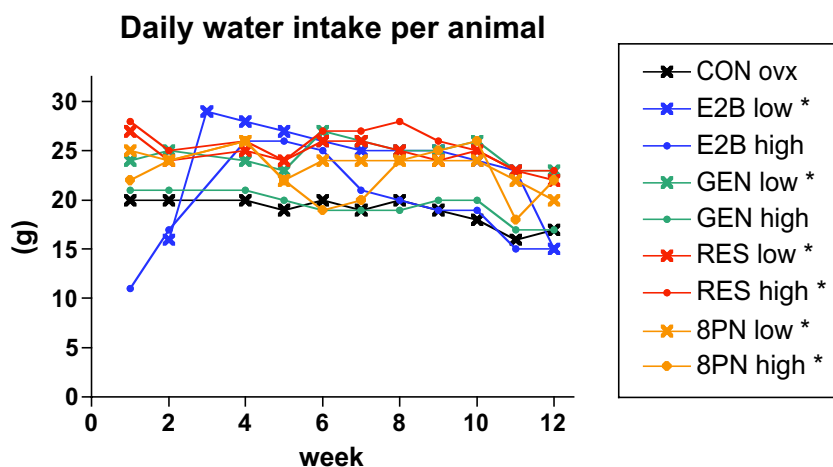
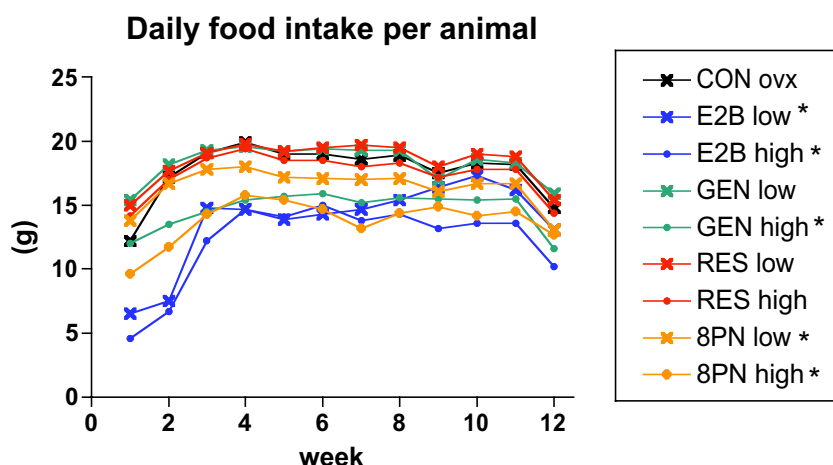
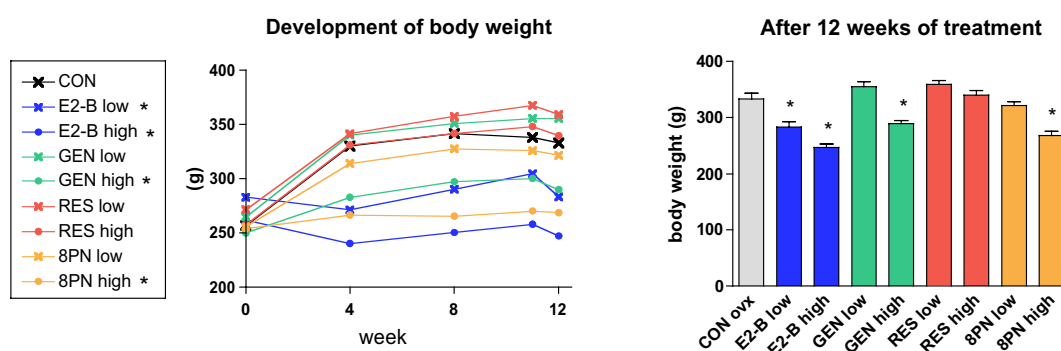


Figure 3-12: Averaged food and water intake per animal/day during 3 months treatment and the correlation of both parameters (\*  $p < 0,05$  vs. CON ovx).

### 3.3.2.2 Body weight development

Every 4 weeks animals were weighed. With the first measurement after ovx the persisting trend for the remaining treatment period was already evident and significant (*Figure 3-13*). Both E2B, GEN and 8PN high dose groups weighed less, which correlated with their reduced food consumption. Starting at a mean body weight of  $260 \pm 1,5\text{g}$  (SEM) they more or less maintained a level within 250-290g. All other groups grew heavier than 300g with up to 370g (RES low), the ovx control ranging around 340g. Weight loss in the last week was attributed to the reduced food intake (see above).



*Figure 3-13: Body weight measurements from ovariectomy throughout the treatment period (\*  $p < 0,05$  vs. CON ovx).*

### 3.3.3 Final dosage

To make proper assumptions on dose-response-correlations it was necessary to obtain information whether the planned dosage was achieved. The factors presented in the previous sections as food content and stability as well as food intake and body weight development of the animals have an influence. Bioavailability, metabolism and tissue distribution affect the final serum levels. Concerning dosage the positive control was always calculated as bioactive E2 instead of the administered E2B ester.

#### 3.3.3.1 Achieved dose

Dose planning was based on experience from earlier experiments calculating with certain ranges of food intake (15-20g/day) and body weight (250-330g). A “dose window” opened, where final doses were aimed at. The actual drug “input” was obtained from food intake measurements and body weights, considering their mean values under the stable conditions of week 4-11.

Table 3-5: Comparison of calculated dose window and actually achieved values.

Daily dose (mg)	AIMED AT		ACHIEVED	
	per animal	per kg BW	per animal	per kg BW
CON	-	-	-	-
E2	low	0,05 - 0,06	0,05	0,17
	high	0,19 - 0,25	0,16	0,70
GEN	low	1,5 - 2,0	1,9	5,4
	high	15 - 20	15,5	53,0
RES	low	1,26 - 1,68	1,6	4,5
	high	12,6 - 16,8	15,3	45,0
8PN	low	1,89 - 2,52	2,1	6,8
	high	18,9 - 25,2	18,4	68,4

Comparing the daily/dose per animal, the achieved values lay within the estimated variation (Table 3-5). The E2B high group ate around 14g/day, while weighing around 250g, which led to a slightly lower exposure. In all groups less food than expected was consumed in comparison to body weight gain. That resulted in decreased doses/kg bw.

### 3.3.3.2 Serum levels

The E2 level in serum reached 40pg/ml (0,15nM) with the low dose, thus reflecting a constant exposure of the animals to E2 peak level during proestrus. With the exposure level of 153pg/ml (0,56nM) the high group switched into the intended pharmacological dose. Control animals remained with ovx reduced E2 level of 10pg/ml, supplied by the minor production in adrenals. These values were obtained in a RIA assay (Figure 3-14 A).

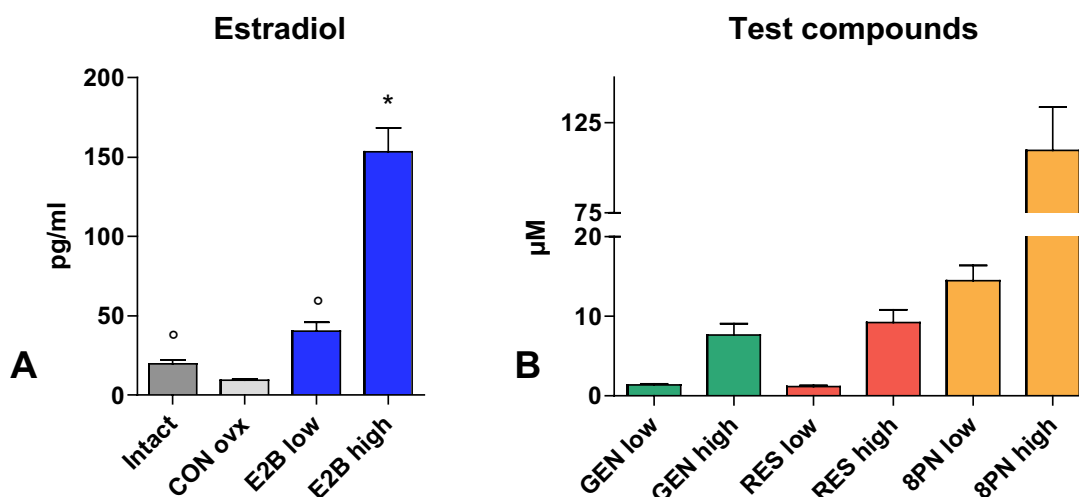


Figure 3-14: Final serum levels of positive control (A) and the equimolar administered test compounds (B) (\* $p < 0,05$  vs. CON,  $^{\circ}p < 0,05$  vs. CON by ANOVA/Dunnnett without E2B high).

Serum levels of the three test compounds are graphed in  $\mu\text{M}$  to simplify comparison after equimolar administration (*Figure 3-14 B*). Low doses of GEN and RES ranged just above the limit of quantitation of the HPLC-UV method used, and their high doses hardly exceeded  $10\mu\text{M}$ . As already seen in the single dose treatment 8PN concentrations lay well above them, here under equimolar conditions by more than factor 10.

The *Table 3-6* provides details on the difference between low and high doses of all substances with intended and actually applied doses, including the detected serum values.

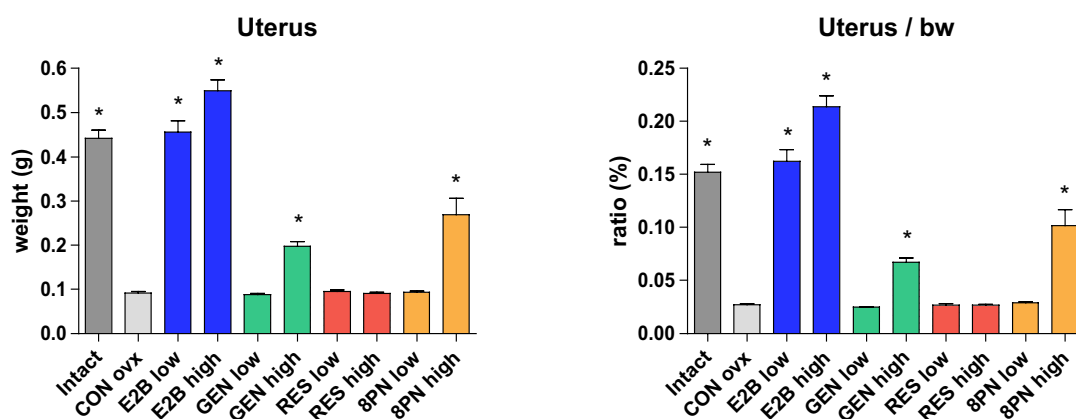
*Table 3-6: Calculated vs. really obtained relation of low and high doses.*

	daily dose	per kg bw	per animal	
	aimed at	achieved	achieved	serum level
E2	1 : 4	1 : 4,2	1 : 3,3	1 : 3,8
GEN	1 : 10	1 : 9,8	1 : 8,2	1 : 5,6
RES	1 : 10	1 : 10,0	1 : 9,6	1 : 7,8
8PN	1 : 10	1 : 10,1	1 : 8,8	1 : 7,6

### 3.3.4 Effects on HPG-axis and reproductive organs

#### 3.3.4.1 Uterus weights

The positive control was the main stimulus for uterus wet weights. *Figure 3-15* shows that the 4x dose difference between the two groups only resulted in a minor weight increase from about factor 5 (E2B low) to factor 6 (E2B high). Intacts ( $0,441\pm 0,019\text{g}$ ) showed nearly the same weight as E2B low ( $0,456\pm 0,025\text{g}$ ). Of the test compounds GEN high doubled, 8PN high tripled uterine weights significantly.



*Figure 3-15: Absolute (left) and relative (right) uterine wet weights after three months treatment via pelleted food (\*  $p < 0,05$  vs. CON ovx).*

### 3.3.4.2 Hormones

The pituitary hormones LH and FSH regulating the gonadal axis were of main interest (Figure 3-16). Their suppression pattern was rather similar, though more pronounced for the more sensitive marker LH. E2B groups dose-dependently suppressed LH and FSH levels though a marginal LH level as in intact was not achieved. LH was also lowered significantly by 8PN high. 8PN shows an increasing tendency of prolactin levels. E2B high nearly tripled the levels of ovx control in contrast to E2B low, that did not evoke major response. Again none of the levels reached intact values, that lay 5 times above CON ovx.

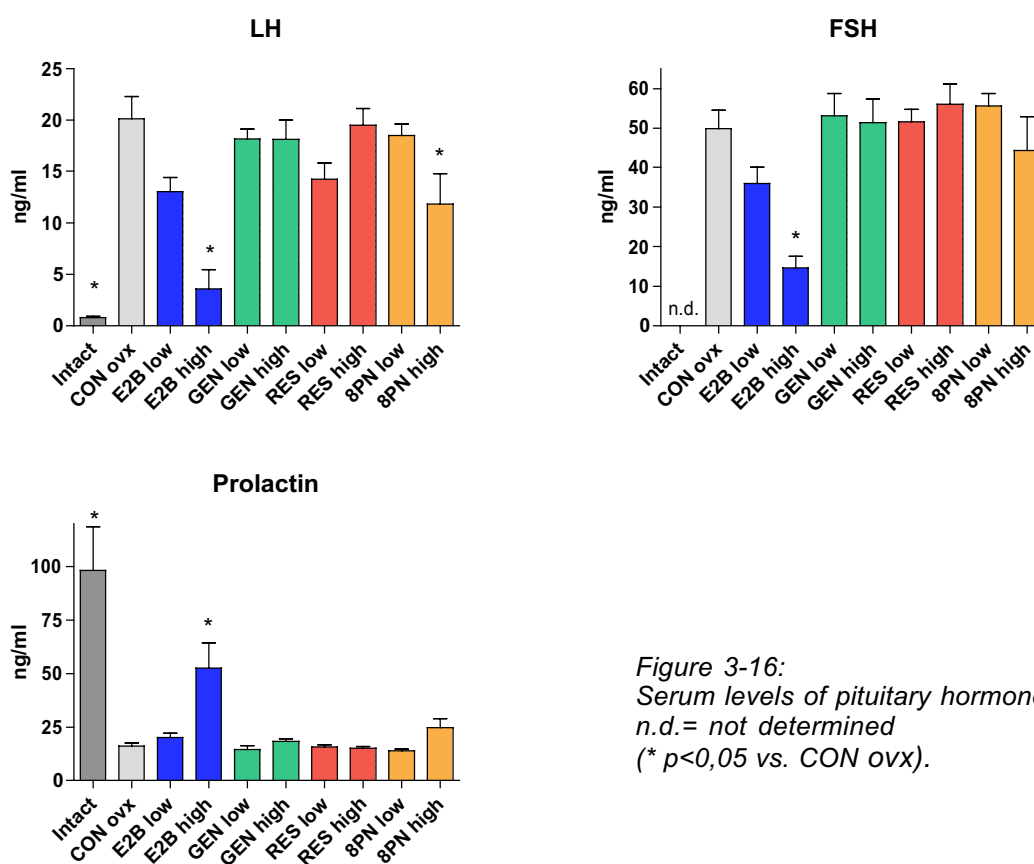


Figure 3-16:  
Serum levels of pituitary hormones,  
n.d.= not determined  
(\*  $p < 0,05$  vs. CON ovx).

### 3.3.4.3 Gene expression

#### Pituitary

The expression pattern of LH $\beta$  and the  $\alpha$  subunit (common to FSH, LH and TSH) parallel the LH serum values quite well (Figure 3-17). The observation of slight though not significant reduction by RES low is repeated in the LH $\beta$ .

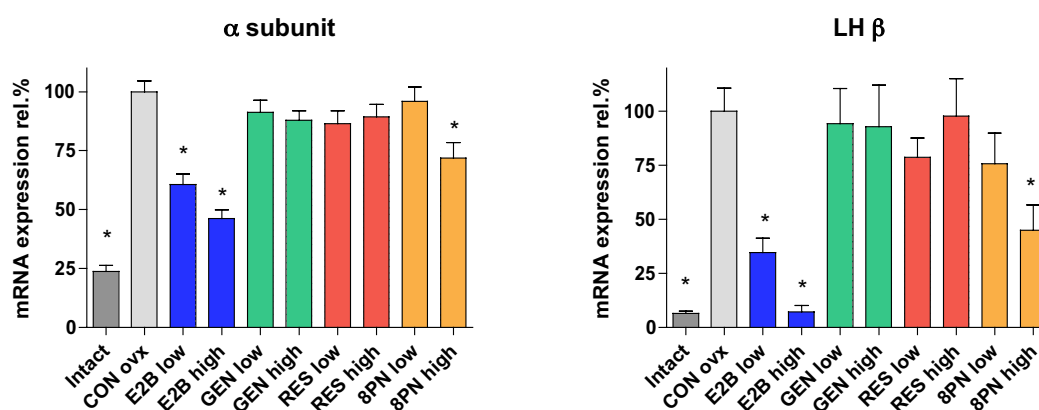


Figure 3-17: Pituitary mRNA expression of  $\alpha$  subunit and LH  $\beta$  (\* $p < 0,05$  vs. CON ovx).

The gene expression of estrogen receptor subtypes  $\alpha$  and  $\beta$  displayed varying pictures (Figure 3-18). ER $\beta$  reflects the distribution found before in LH and FSH serum levels as in pituitary LH gene expression. ER $\alpha$  seemed induced by the low dose of E2B, while it was significantly reduced in intact animals.

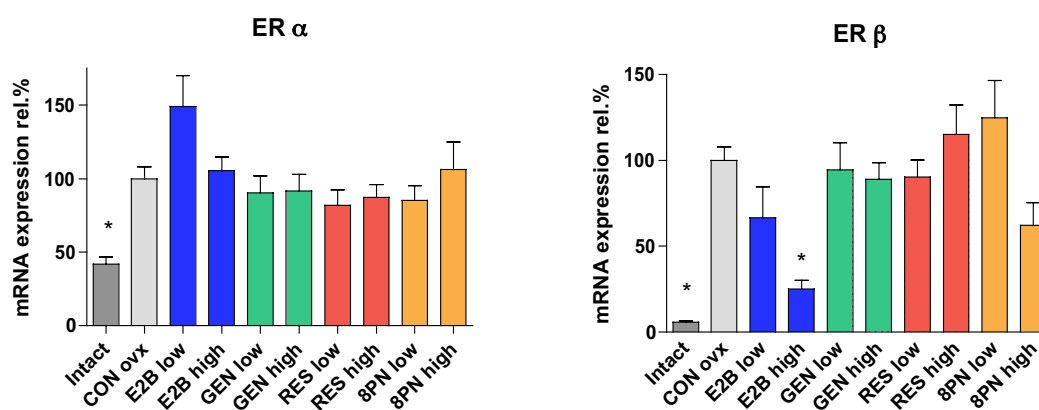


Figure 3-18: Pituitary mRNA expression of ER subtypes  $\alpha$  and  $\beta$  (\* $p < 0,05$  vs. CON ovx).

## Uterus

As classical marker genes for estrogenic action in the uterus expression levels of IGF1, C3 and PR were analyzed (Figure 3-19). Activation of the C3 gene was most sensitive and expression matched the previously observed uterine weights in treated animals, with strongest induction by E2B though not showing dose correlation and slighter effects of GEN high and 8PN high. The intact group achieved levels comparable to E2B low though uterine weights were much less stimulated. IGF1 and PR genes were mainly activated by E2B treatment lacking conclusive effects by the test compounds. In intact PR transcripts were similar to E2B high, while showing an extraordinary stimulation of IGF1.



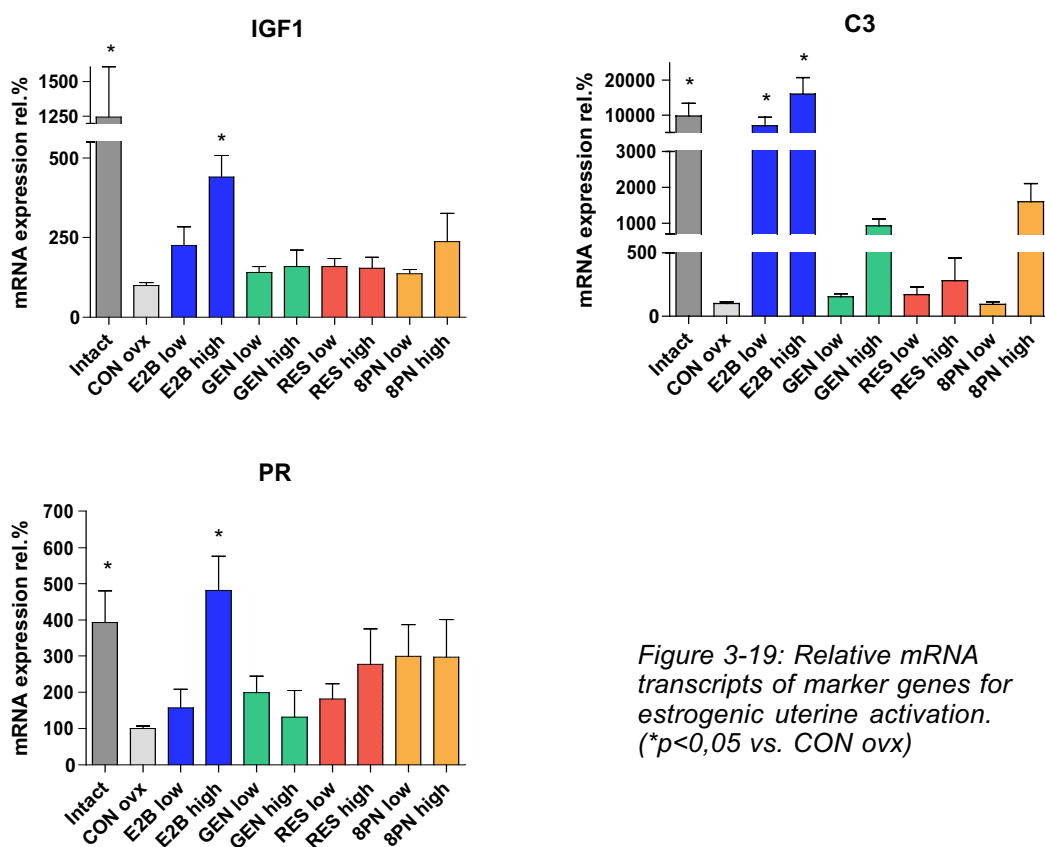


Figure 3-19: Relative mRNA transcripts of marker genes for estrogenic uterine activation. (\* $p < 0,05$  vs. CON ovx)

The analysis of ER $\alpha$  and ER $\beta$  showed no statistical significances, but diminished activation by E2B low and high, GEN high and 8PN high could be observed (Figure 3-20).

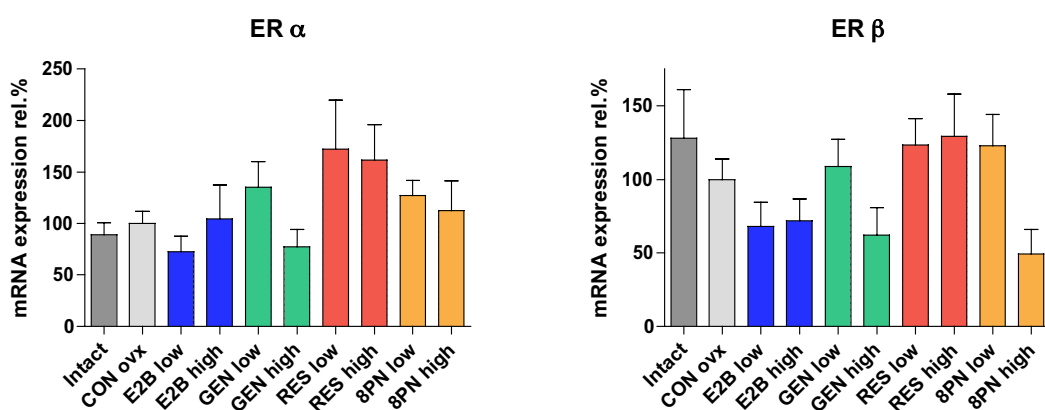


Figure 3-20: mRNA expression patterns of ER subtypes in uterus (\* $p < 0,05$  vs. CON ovx).

### 3.3.4.4 Histology

#### **Uterus**

The analysis criteria are explained in the histology section 2.9.3 Analysis p.50 *Figure 2-17*. The pictures relating to the following descriptions can be viewed on the page after in *Figure 3-21*. The original photographs of the left column were taken with 20x, the enlarged view of a selected area in the right column with 40x magnification.

- CON** The endometrium was composed of cuboidal inactive cells. The connective tissue looked as an unorganized lax syncytium of round nuclei. No mitotic activity was detected.
- E2B** Both E2B doses affected stromal cells, which adopted spindle shape. Hypertrophic and hyperplastic glands were detected in half of the animals of low and all of high dose. E2B high also induced squamous metaplasia, detected in some animals, and pyometra in half of the animals. Mitotic activity was present and erratic in both doses.
- GEN** With the low dose only lamina propria cells were affected in half of the animals, while the high dose affected them in all. Endometrial mitotic activity was absent in GEN low and detectable in 3 animals of GEN high.
- RES** In both RES groups lamina propria cells showed spindle shape regardless of dose in around half of the animals.
- 8PN** Lamina propria spindle cells were found in 3 of low and all animals of high dose. 8PN high also showed hyperplasia and hypertrophy of endometrial glands in nearly all and squamous metaplasia in more than half of the animals. Mitotic activity was not detected in 8PN low and seen in two animals of 8PN high. The high dose also induced a different lesion unique to this group: Polypoid projections into the uterine lumen were found in 6/10 samples. A unique example of them is shown (originally 10x magnification).

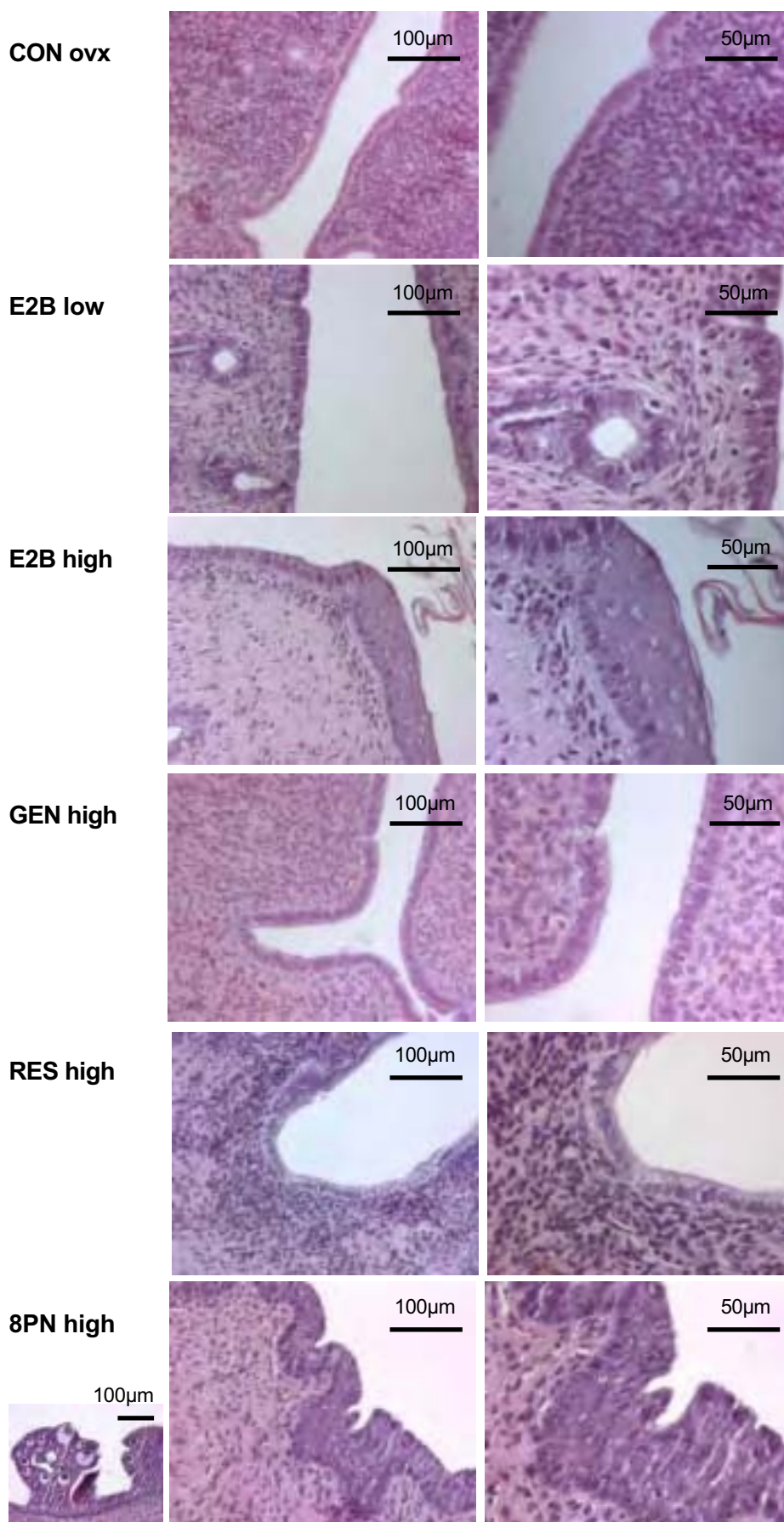


Figure 3-21: Appearance of uteri after 3 months treatment (transversal sections, HE stain).

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## Vagina

The analyzed features are described in the histology chapter 2.9.3 Analysis p. 51.

In *Figure 3-22* pictures of sections (original photographs 20x) are shown on the left and of smears on the right (originally 40x magnification).

- CON** Smears: In CON group few small basal cells with large nuclei were found.  
Sections: Vaginal epithelium was atrophic. In all animals only 2 to 3 cell layers were seen, composed of flattened cells without cornification.
- E2B** Smears: Cells were more abundant in E2B high, also retained nuclei were common in contrast to complete absence in E2B low. Inflammatory polymorphonuclear (PMN) cells were seen abundantly in 4/11 smears belonging to animals that developed pyometra.  
Sections: The epithelium adopted a typical squamous multi-layered appearance. Around 10 cell layers with cornification were seen in E2B low, and a higher number of epithelial layers from 10 to 15 were found in E2B high.
- GEN** Smears: GEN low displayed features like CON, except additional appearance of some PMN cells. GEN high showed a mixed pattern: Large polygonal cells with condensed nuclei were not cornified, while medium sized cells with still large nuclei showed partially cornified cytoplasms.  
Sections: The number of cell layers in GEN low did not differ from controls and was increased to 5 to 10 in GEN high. Mild cornification was found only in one animal of GEN high. Cytoplasmatic vacuolization of epithelial cells was seen incipient in 5/12 animals of GEN low and all samples of GEN high.
- RES** Smears: The low dose of RES looked like CON. In RES high basal cells were increased to medium size without any sign of cornification, and some PMN cells could be found.  
Sections: No differences to controls were seen. 2-3 cell layers were found without cornification.
- 8PN** Smears: Under 8PN low nuclei were enlarged and cytoplasms were foamy and unstained. In 8PN high smears cells were increased in size, held large active nuclei and mainly maintained round shape. Some of these cells showed incipient cornification. The few large polygonal cells, that were seen, were never cornified and retained nuclei. Under this treatment cells with vacuoles were detected in the smears.

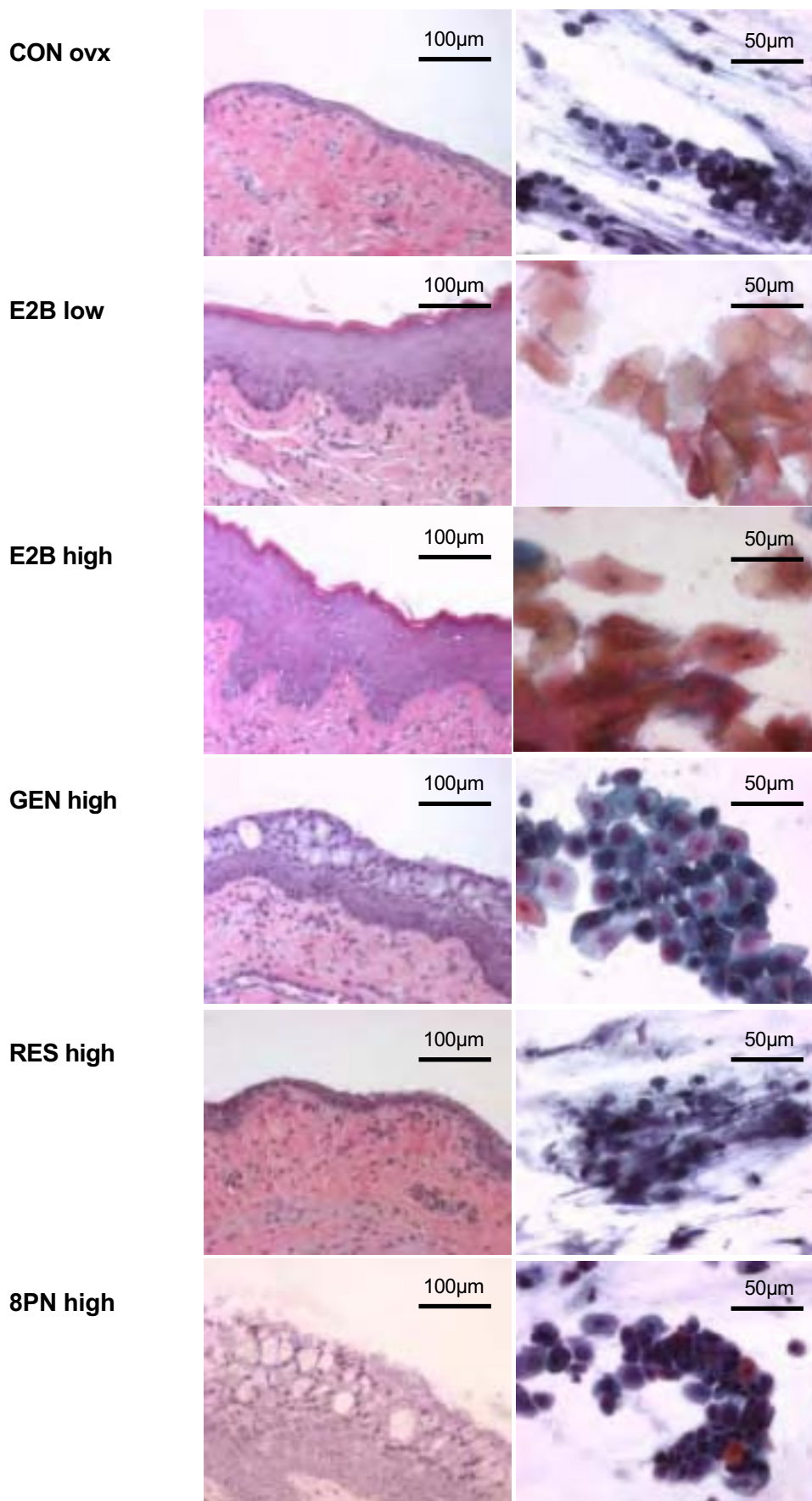


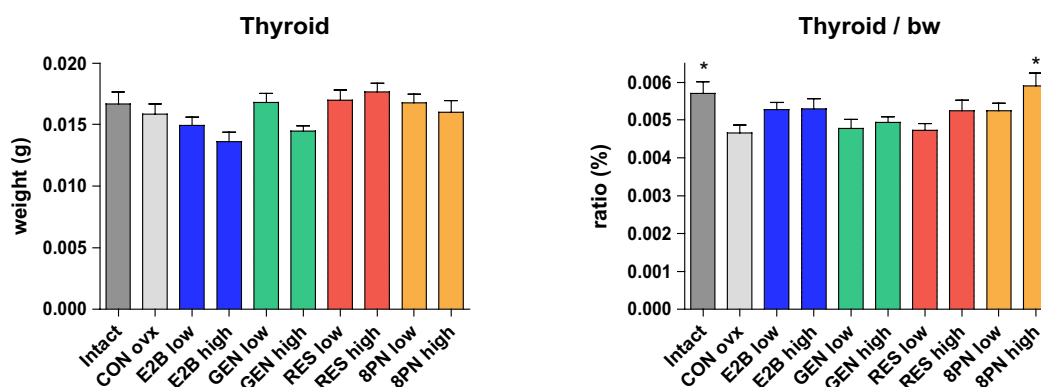
Figure 3-22: Sections of vaginae, HE stain ( left), Vaginal smears, PAP stain (right).

**8PN** *Sections:* The low dose resembled control group, although some areas showed up to 4 layers. In 8PN high all animals had a multilayered epithelium. The number of cell layers was difficult to interpret due to the unorganized pattern and variability between the animals, ranging from 3 to 15. Typical squamous multi-layered organisation was found in 3/10, although no cornification was seen and the upper layers conserved nuclei. Intraepithelial vacuoles were incipient in half of samples of 8PN low and one animal of 8PN high. All other animals of 8PN high showed clear vacuolization.

### 3.3.5 Effects on HPT-axis

#### 3.3.5.1 Thyroid weights

No significant changes appeared for absolute thyroid weights as can be seen in *Figure 3-23* on the left. Considering relative thyroid weight dose independent rise by E2 could be observed, as well with RES high. Significantly higher weight showed intact and 8PN high.



*Figure 3-23: Absolute and body weight related weights of the thyroid (\* $p < 0,05$  vs. CON ovx).*

#### 3.3.5.2 Hormones

Variation of effects was high for serum levels of TSH and free and total amounts of T3/T4 (*Figure 3-24*). Clearly the free T4 levels were not altered by any treatment. Intact and CON showed similar levels of hormones besides total T3. The E2 doses always resulted in biphasic pattern, either one showed a tendency, while the other did not, or both even displayed completely opposite trends. Of the test compounds GEN reduced free T3 levels, and RES high and both doses 8PN caused increase of total T3 levels.

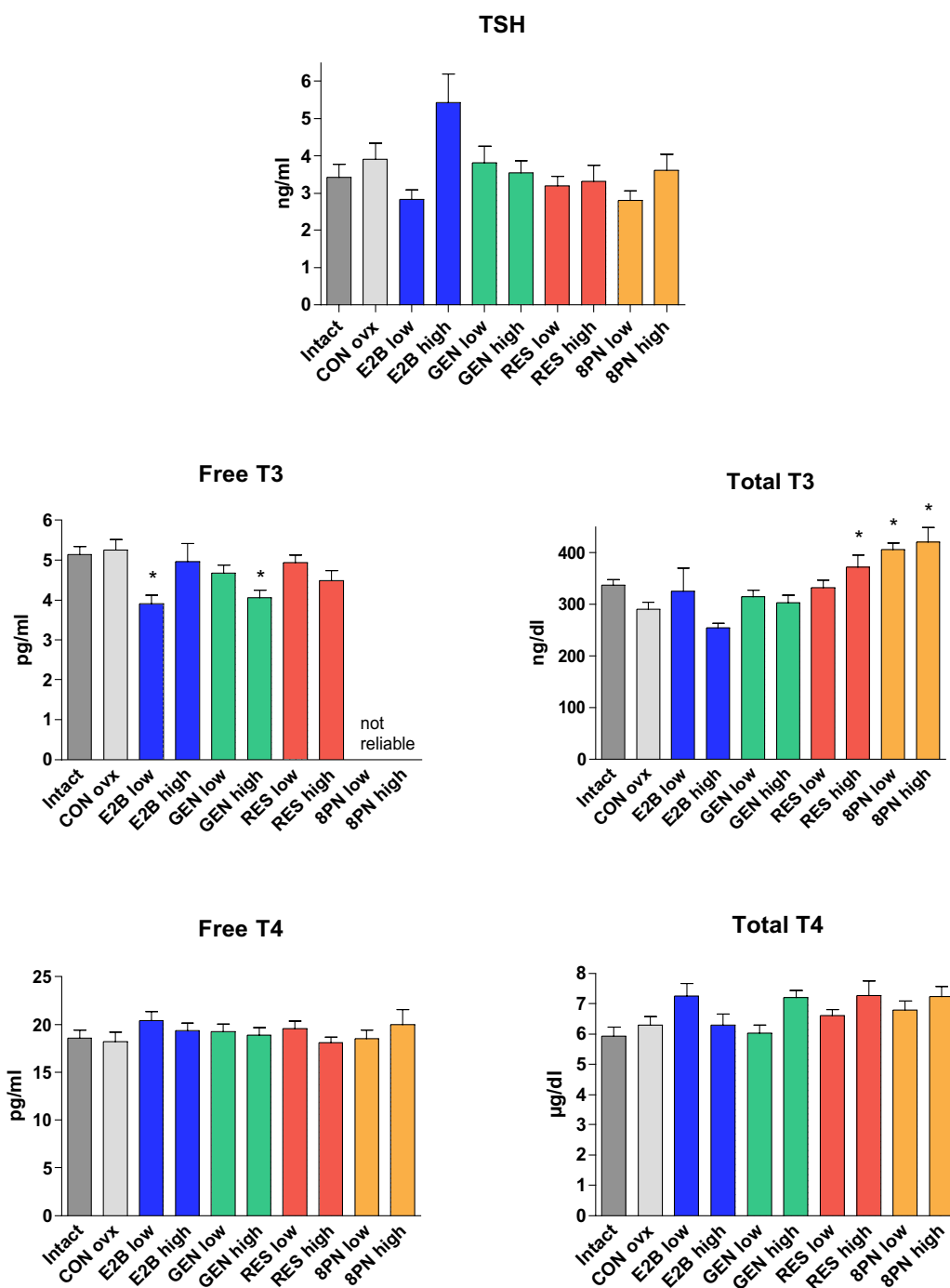


Figure 3-24: Serum levels of pituitary and thyroid hormones regulated within the HPT-axis after three months treatment (\* $p < 0,05$  vs. CON ovx).

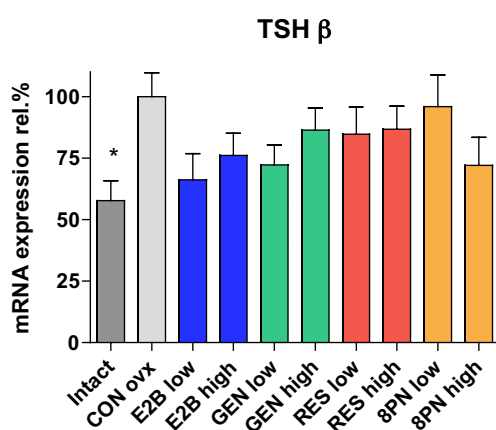
When relations of hormones were compared (*table 3-7*), the same trends could be observed: RES high altered free/total T3 relation, which was due to the increase of total T3. The effect of E2B low and GEN high on free/total T3 and free T3/T4 relations was caused by their reduction of free T3.

*Table 3-7: Proportions of free and total fractions and among the thyroid hormones T3 and T4. (\*p<0,05 vs. CON)*

relation %	intact	CON	E2B		GEN		RES		8PN		
			low	high	low	high	low	high	low	high	
free/total	T3	0,15	0,18	0,12	0,20	0,15	0,13	0,15	0,12		
	T4	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,03	0,03
T3/T4	free	27,7	28,8	20,3	25,7	24,2	21,5	25,2	24,8		
	total	5,7	4,6	5,0	4,0	5,2	4,2	5,0	5,1	6,1	5,8
<b>% of CON</b>											
free/total	T3	84	100	66*	108	82	74*	82	67*		
	T4	108	100	101	106	110	91	103	86	96	96
T3/T4	free	96	100	71*	89	84	75*	87	86		
	total	123	100	108	88	113	91	109	111	131	126

### 3.3.5.3 Gene expression

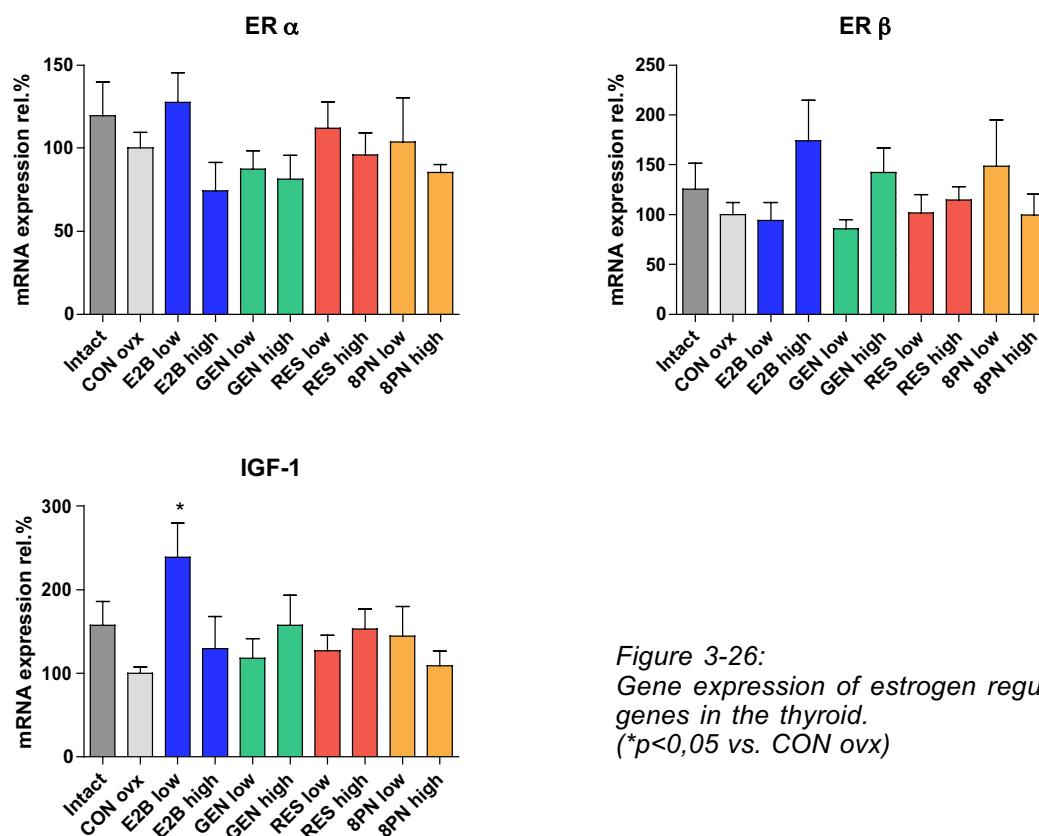
The pituitary mRNA-expression of the specific subunit  $\beta$  of TSH corresponded with the determined TSH serum levels of E2B low, but contrasted with the level of E2B high found in serum. Except the low expression in intact no significances were observed (*Figure 3-25*).



*Figure 3-25: Relative transcripts of the specific TSH  $\beta$  in the pituitary (\*p<0,05 vs. CON ovx).*



In the thyroid relative transcripts of IGF1 and ER $\alpha$  were upregulated in intact and E2B low animals, without any remarkable effects by any other treatment. ER $\beta$  slightly responded to E2B high, and was possibly induced by GEN high (*Figure 3-26*).



*Figure 3-26:*  
Gene expression of estrogen regulated genes in the thyroid.  
(\* $p < 0,05$  vs. CON ovx)

### 3.3.5.4 Thyroid histology

Of all groups, except low doses of GEN, RES and 8PN, 3 animals that were most affected in serum levels of TSH, T3 and T4 were selected for initial histological inspection.

For the description and explanation of analyzed features please refer to the histology section 2.9.3 Analysis p. 52.

Variability of colloid size was large among every group, while number and shape were not affected. Thyroglobulin storage within the colloids made visible by PAS stain was also widely differing within each group. Appearance of follicular cells lining the colloid was not affected either. Thus by the selected parameters effects of long term treatment were not detectable. Representative pictures can be viewed in *Figure 3-27*.

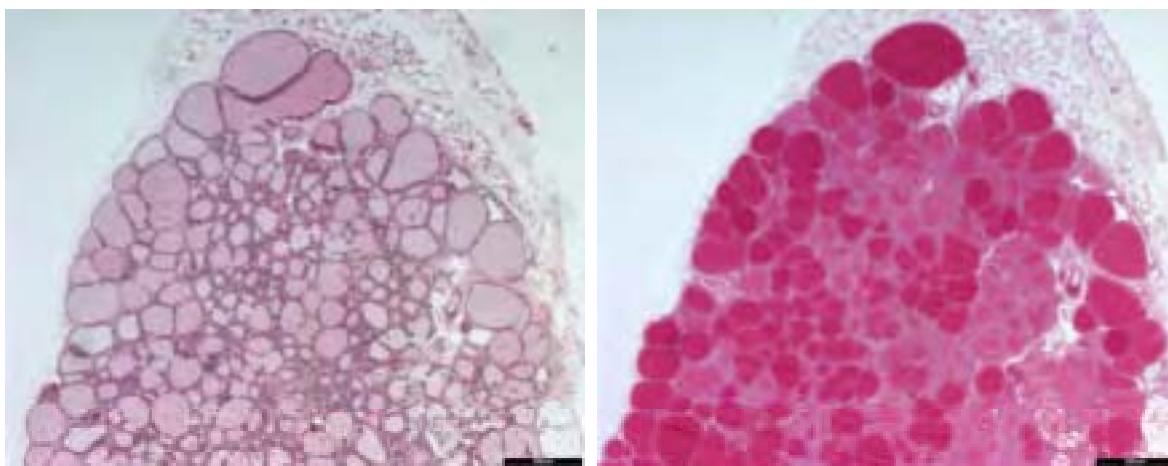


Figure 3-27: Appearance of in all groups unaltered thyroid after three months treatment with different staining techniques, consecutive sections of the same sample stained with HE (left) and PAS (right). Original magnification 5x.

### 3.3.6 Target organs of estradiol and thyroid hormone action

#### 3.3.6.1 Liver

##### Liver weight

Absolute liver weights showed no significant effects (Figure 3-28), besides decreasing trend in GEN high and 8PN high groups. Relative weights of E2B treated animals were elevated significantly, of intact animals slightly. Test compounds did not affect relative weights.

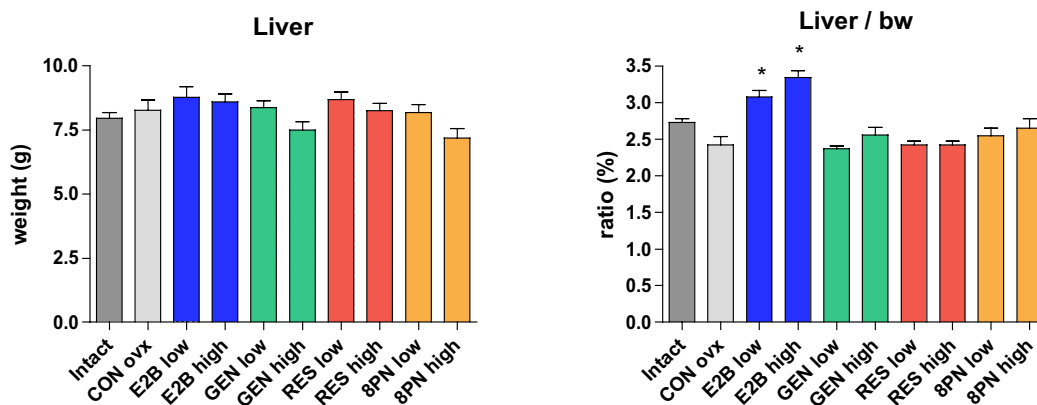
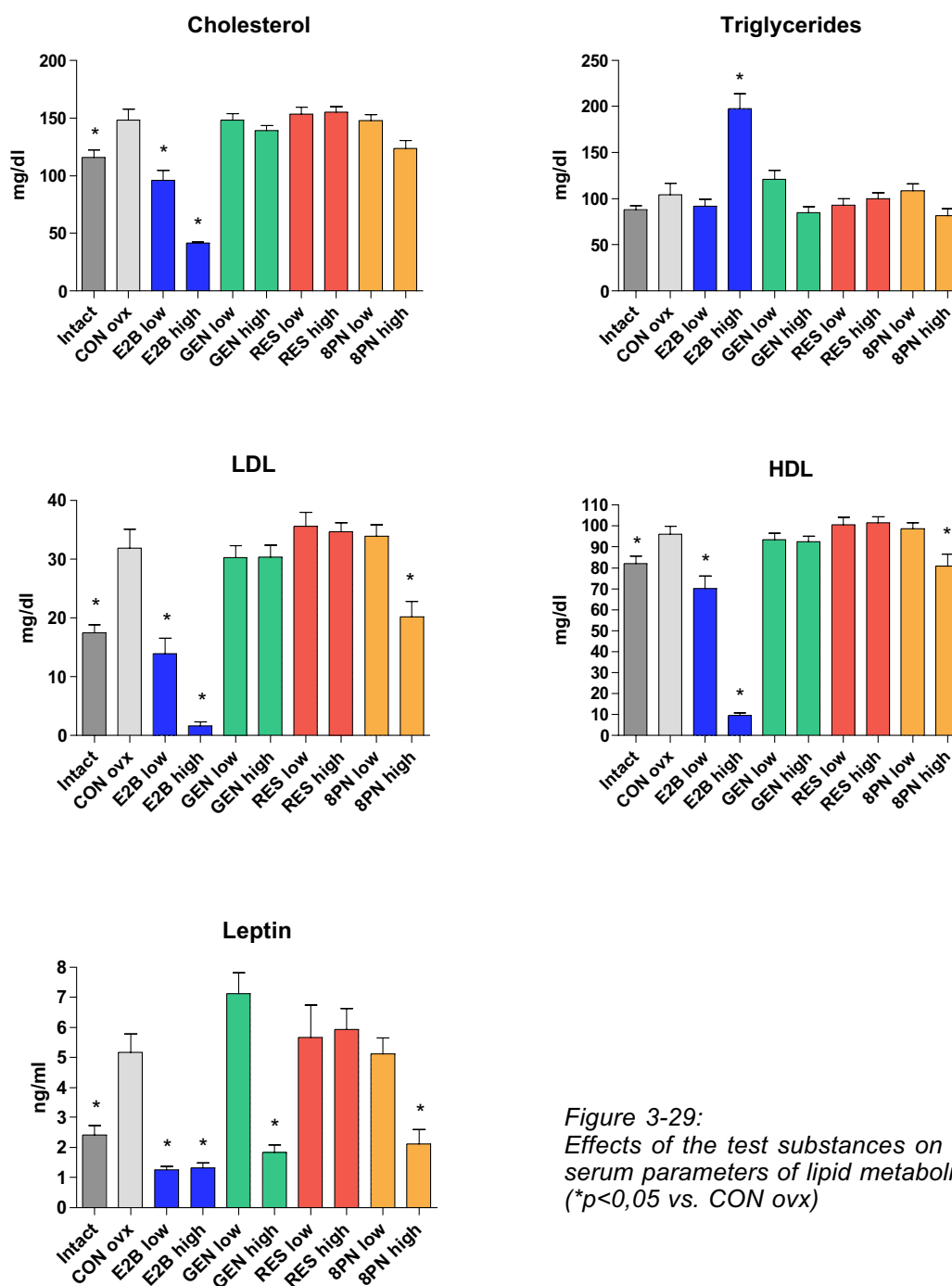


Figure 3-28: Comparison of absolute and relative liver weight after 3 months treatment. (\* $p < 0,05$  vs. CON ovx)

### Metabolic parameters in serum

Analyzing lipid metabolism cholesterol, HDL and LDL were significantly reduced in intact and both E2B treated animals (*Figure 3-29*). E2B high always exerted the strongest effect, while intact and E2B low displayed the same strength. 8PN high also showed decreased levels, though much less pronounced than positive control groups. The classical rise of triglycerides by E2 was only achieved by the high dose of E2B. Leptin was mostly sensitive to the treatments and was similarly suppressed in intact, both E2B, GEN high and 8PN high groups to one third to one quarter of CON ovx group.



*Figure 3-29:*  
Effects of the test substances on serum parameters of lipid metabolism. (\* $p < 0,05$  vs. CON ovx)

### Gene expression

Figure 3-30 shows that no significant alterations of mRNA expression of IGF1 and ER $\alpha$  genes could be detected. Expression of ER $\beta$  was too low to be determined with our system.

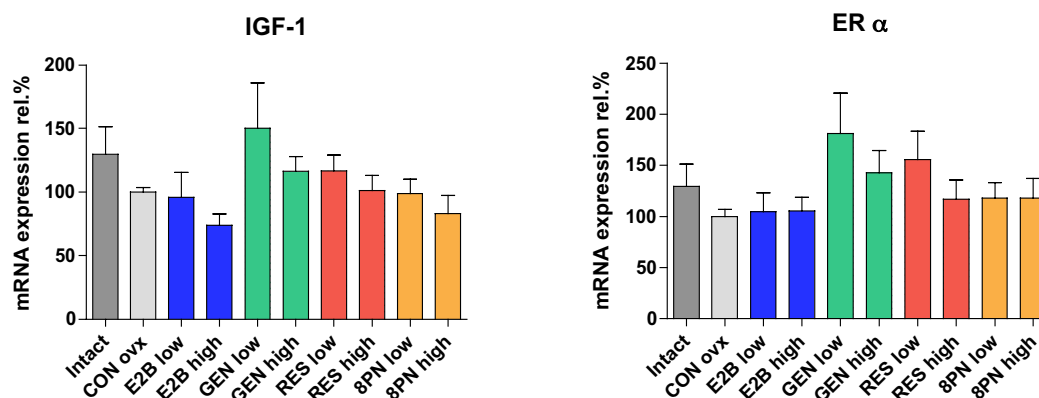


Figure 3-30: Relative mRNA transcripts of estrogen regulated genes in the liver. (\* $p < 0,05$  vs. CON ovx)

### 3.3.6.2 Bone

#### Bone metabolism markers in serum

The osteoblast activity marker protein osteocalcin was not altered between intact, CON and E2B low, while E2B high significantly reduced its serum levels (Figure 3-31). 8PN high also showed a tendency of decline. On the other hand osteoclast activity, which was measured by the collagen fragment RatLaps, was only induced by E2B low, without any effects by the other treatments.

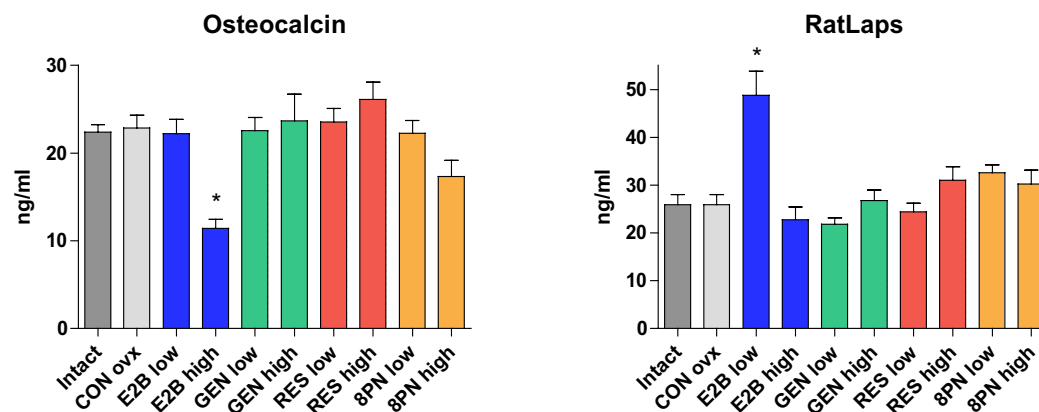
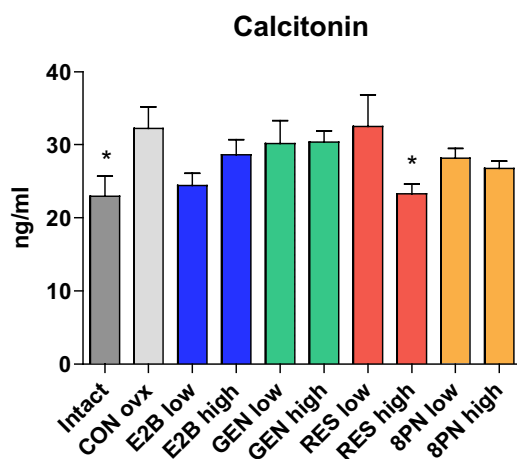


Figure 3-31: Serum levels of markers of bone turnover. (\* $p < 0,05$  vs. CON ovx)

Thyroid secretes calcitonin, which inhibits osteoclast activity resulting in decrease of calcium mobilization from the bone. The E2B low, intact and RES high groups showed a decline of calcitonin serum levels, that was significant for the latter two (*Figure 3-32*). E2B high and the 8PN groups seemed to have a slight decreasing effect as well.



*Figure 3-32:*  
Serum levels of the C-cell derived thyroid hormone calcitonin. (\* $p < 0,05$  vs. CON ovx)

### Computer tomographic parameters

Trabecular density ( $\text{mg}/\text{cm}^3$ ), total mass (mg) and strength strain index ( $\text{mm}^3$ ) are shown in %-relation (*Figure 3-33*).

The most prominent change during bone resorption due to lack of E2 is the drastic decline of trabecular density. While the density was maintained in the intact animals, it dropped through ovariectomy to less than 50%. E2B substitution was able to compensate and keep the density at 87% (E2B low) and 74% (E2B high). GEN high and 8PN high also increased the density to 63% and 64% respectively. Low doses of these two substances were not able to cause any positive effect, as none of the RES doses.

Variation of the total mass and strength strain index is not as strong as of trabecular density, and they display the same effects. The test compounds did not show much difference vs. CON, ranging close to the start value. In intact and E2B low groups the SSI was improved by about 10%, but declined in the E2B high group by more than 15% taking CON as reference.

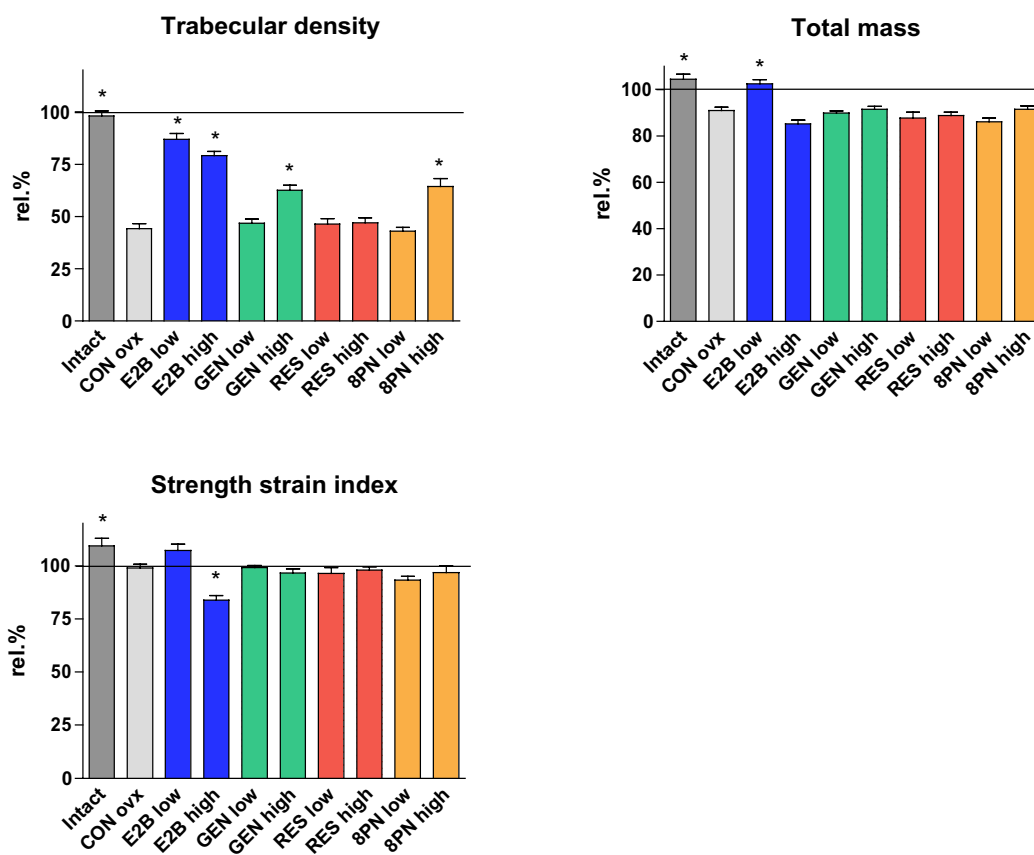


Figure 3-33:  
CT derived parameters indicating bone condition. Final values are %-related to the overall mean at the start of 3 months treatment, which is marked by the line.  
(\* $p < 0,05$  vs. CON ovx)

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## 4 Discussion

### 4.1 Final dosage

The determination of the applied dosage in the long term treatment bears the difficulties of any food study. A deviating content of test compounds in the diet from the calculated dosage due to the manufacturing process or stability problems impairs exact specification of the concentration in the food and requires its close monitoring. Therefore the serum levels of the test compounds were considered for the comparison of in vivo effects.

The concentration of the test compounds in the pituitary cell culture system was 1µM. It resulted in significantly increased cell viability by all substances except RES, that parallels their degree of estrogenic potency. In vivo total serum levels (i.e. after incubation with β-glucuronidase and sulfatase) were measured. Therefore it has to be kept in mind that the determined values include phase II metabolites. The single dose challenge with 50mg per animal achieved blood levels of approx. 25µM (GEN), 100µM (RES) and 200µM (8PN) respectively. After equimolar administration in the long term experiment both GEN and RES reached approx. 1µM with low doses and 10µM with high doses, whereas 8PN achieved serum levels that were tenfold higher amounting to 10 and 100µM.

Factors that influence the serum levels achieved by any substance are tissue distribution, bioavailability and metabolism.

For GEN and RES several studies have been conducted to investigate these aspects. An absolute oral bioavailability of 15% was determined in female rats with radioactively labeled GEN (Coldham *et al.* 2002) and, also in rats, 38% bioavailability are published for RES (Marier *et al.* 2002). After absorption GEN is extensively metabolized in phase I (Kulling *et al.* 2000) and before being glucuronidated in phase II metabolism, while RES immediately undergoes glucuronidation and sulfation (Yu *et al.* 2002; Kaldas *et al.* 2003). In rats half time of GEN and metabolites is approx. 8,5h (females) (Coldham *et al.* 2000), that of RES and its glucuronide even shorter with approx. 1,5h, but enterohepatic recirculation was shown after 4-8h (Marier *et al.* 2002). A study on the metabolism of 8PN with human liver microsomes was published only recently (Nikolic *et al.* 2004), where at least 12 metabolites of phase I and II were identified. Tissue load was tested with 14C-RES p.o. and postulated for organs of (re)absorption, metabolism and elimination, namely liver and kidney (Vitrac *et al.* 2003). In female rats orally applied 14C-GEN persisted in liver and reproductive organs, in uterus mainly as parent compound (Coldham *et al.* 2000). For 8PN no such information exists yet, but an affinity to organs of high ER $\alpha$  expression can be assumed.

On the other hand in the presented study a concentration of 8PN in serum was found that was ten times higher than that of GEN and RES. Since those two are subjected to fast metabolism this suggests that only a minor fraction of 8PN is actually metabolized in vivo. The total GEN levels achieved in the long term treatment are in good accordance with the values from literature that were the base for the choice of overall dosage (Doerge *et al.* 2002a) (see Introduction 1.5 Long term treatment p.16). Thus, the doses applied to the animals actually resemble human exposure levels to GEN and RES and conclusions from the long term experiment seem likely for estimation of their potential benefit and risk. The 8PN doses reached effective serum levels in the present experiment and the results contribute to safety assessment.

## 4.2 Effects on HPG- and HPT-axes in the three experimental setups

### 4.2.1 HPG-axis

Stimulation of GnRH-induced LH release in anterior pituitary cells clearly indicates estrogenic activity of the substance in the organ, even more if the increase can be antagonized by the antiestrogen ICI 182,780.

In vivo the negative feedback of E2 on the pulsatile pituitary LH secretion reduces the activity of the hypothalamic GnRH pulse generator. Overactivity of the pulse generator during the climacteric years produces an overflow of neurotransmitters to neighboring neurons, that regulate temperature and heart beat rate (Tataryn *et al.* 1979; Gambone *et al.* 1984). This causes hot flushes associated with tachycardiac attacks. LH suppression caused by estrogenic substances promises alleviation of these complaints.

All compounds except RES displayed antagonizable estrogenic effects on LH release in the pituitary cell culture, though differing in extent of stimulation. The activity of the test substances varied in the in vivo experiments with ovx rats.

GEN showed a lowering effect after challenging the animals with a single dose, which in contrast was not found any more after 3 months treatment. LH levels were not affected by RES in any experiment. 8PN reduced LH in the single dose treatment and kept that effect in the long term treatment, just as E2/E2B, which suppressed LH levels most strongly and dose dependently. None of the treatments however, achieved LH levels as low as observed in intact animals. The compilation of treatment and effect variation together with exposure levels of the test compounds allows further interpretation (*Table 4-1*), since the dosage is another factor for effective treatment.



Table 4-1: Treatment and dose dependent effects on LH release in pituitary cell culture (=in vitro) and after oral treatment of ovx rats with a single high dose (=1x) or after 3 months treatment with 2 doses (=low + high).

↑↓=significant changes

(in vitro vs. CON, 1x vs. baseline values before treatment, low+high vs. CON ovx)

	E2		GEN		RES		8PN	
	conc.	LH	conc.	LH	conc.	LH	conc.	LH
<b>in vitro</b>	1 nM	↑	1 µM		1 µM		1 µM	↑
<b>in vivo 1x</b>	1,2 nM	↓	25 µM	↓	100 µM		200 µM	↓
<b>low</b>	0,15 nM	↓	1 µM		1 µM		10 µM	
<b>high</b>	0,56 nM	↓	10 µM		10 µM		100 µM	↓

The ability of GEN to diminish hot flushes is still under debate and did not prove to be really effective in some human studies (Burke *et al.* 2003; Tice *et al.* 2003). The results of the 3 months treatment suggest once more that long term intake may not improve symptoms. The ability to affect LH release in the ovx rat requires a dosage of at least 25µM, which is not achieved with the serum levels resulting from exposure to an elevated dose (GEN high) in humans.

The claims for RES in menopausal preparations must be dismissed. Anyway its estrogenicity is fairly weak (Mueller *et al.* 2004), and a reduction of LH would be surprising. The antiestrogenic properties in presence with E2, which in ovx animals is still present at very low levels, might also be responsible for the lack of effects.

With the animal model used, it remains unclear which problems the use of RES might cause in premenopausal women with higher E2 levels, or when it is taken on aside for its antioxidative properties while under hormone therapy.

Best candidate of a compound with therapeutic potential for hot flushes is 8PN. The LH suppression remained unaltered from the single dose administration to the three months treatment with 8PN high, though the low dose was ineffective. The 10 times higher serum concentration in comparison to GEN may be the advantage of 8PN concerning pituitary effects. A major factor of its stronger influence on LH release is probably the estrogenic potency of 8PN. In the Ishikawa cell assay EC<sub>50</sub> values were 4 vs. 200nM (E2 0,8nM) and in a yeast screen 40 vs. 1200nM (E2 0,3nM). The relative binding affinity of 8PN lay nearly 8 fold above that of GEN when tested with rat uterine cytosol (Milligan *et al.* 1999).

On the other hand GEN and 8PN increased uterine weight after the single dose (probably due to hemodynamic changes), and after long term exposure (morphological changes). Uterotrophic effects are undesired since they may increase the risk of uterine cancer.

It is important to note, that after long term treatment with high doses of the two substances weights lay even above those of intact animals, though still lower than under E2B substitution. This calls for caution where GEN is proposed for treatment of menopausal symptoms, and the results need to be confirmed in preclinical toxicity tests.

The additional information of the long term experiment about FSH release and modulation of LH gene expression supported the picture already displayed by serum LH. Secretion of prolactin, which is coregulated by E2 and T3 (DiPippo *et al.* 1995), seems to require a specific stimulus that only E2B at physiological levels could provide, because even the potent 8PN only caused a minor increase. Though the three test compounds are known to bind to ER $\alpha$  and ER $\beta$ , ER $\alpha$  expressing cells in the pituitary seem not equally susceptible to the ligands because only physiological E2 levels were able to induce a slight increase of gene expression, while ER $\beta$  transcription appears inducible as indicated by dose dependent reduction through E2B and 8PN high.

#### 4.2.2 HPT-axis

The interpretation of the effects of the test compounds on the thyroid axis is difficult. Many studies were performed in vitro and in vivo to illuminate its complex function. Thyroid hormones are not released in pulses, but constant levels of their free fractions are essential for biological function (Ostrowska *et al.* 2003), and various regulatory mechanisms help to maintain equilibrium. TSH levels vary under E2 influence (Donda *et al.* 1990). E2 is known to modulate the thyroid gland during a hormonal cycle, to increase organ size and upregulate its own receptors within the organ (Banu *et al.* 2002). This could be observed after the long term exposure with increased relative organ weights and higher extent of gene expression of IGF1, ER $\alpha$  and ER $\beta$  in intact and E2B treated when compared to the ovx animals. It also affects the conversion of T4 to T3 by 5'-iodothyronine deiodinases (5'-DI) in pituitary, liver and thyroid (Lisboa *et al.* 1997). Flavonoids not only inhibit 5'-DI, but also the activity of thyroid peroxidase in follicular cells (Aufmkolk *et al.* 1984; Divi *et al.* 1997; Ferreira *et al.* 2002). A synthetic flavonoid was shown to displace T4 and T3 from transthyretin in the rat, where this is the major carrier protein in serum, necessary to sustain a sufficient pool and make thyroid hormones available at any time (Köhrlé *et al.* 1989). Thyroid receptor interaction may play a role as well (DeVito *et al.* 1999).

In the pituitary cell culture 1nM E2 caused an effect on TSH release which was antagonizable with the antiestrogen ICI 182,780 and thus showed a similar effect as 8PN did in a 1 $\mu$ M concentration.

E2B, 8PN and GEN elevated TSH also in vivo after the single dose, but this effect was lost after 3 months exposure, where only the supraphysiological dose of E2 showed a trend to elevated TSH concentration. Neither in vitro nor in vivo RES provoked any considerable response in TSH release.

Table 4-2 shows a compilation of effects on thyroid hormones in both in vivo experiments. The thyroid hormones in serum 6h after the oral single dose administration were significantly affected by E2 and RES. Even after this brief period changes of T3 and T4 levels can be detected in rats because the half lives of both hormones are much shorter than in humans, namely 6h for T3 (vs. 1d in humans) and 12-24h for T4 (vs. 5-9 days) (Choksi *et al.* 2003). E2 lowered the free and total fraction of T3. RES decreased free and total levels of both hormones leaving the ratio of free to bound unaffected.

Table 4-2: Overview of effects in HPT-axis after oral treatment of ovx rats with a single high dose and after 3 months treatment with a low and high dose of realistic exposure level. CON ovx and intact rats did not show notable changes after the long term treatment. Alterations vs. CON ovx: ↑↓=significant changes, ↑↓=trends

	TSH	FT3	FT4	TT3	TT4
E2 1x	↑	↓		↓	
E2B low		↓			↑
E2B high	↑				
GEN 1x	↑	↓			
GEN low					
GEN high		↓			↑
RES 1x		↓	↓	↓	↓
RES low					
RES high				↑	↑
8PN 1x	↑				
8PN low				↑	
8PN high				↑	↑

At the end of 3 months exposure to GEN high a decline of free T3 was found, which could only marginally be seen after the single dose administration. The same effect of declined T3 persisted with the physiological dose of E2B. Interestingly, free hormone levels of T3 and T4 in CON ovx and intact animals were similar.

In contrast to the overall lowering effects of the single dose administration of the test compounds RES increased total T3 levels in the long term. 8PN elevated total T3 as well and even more evident. An increase of total thyroid hormones is reported under HRT due

to elevated protein levels (Choksi *et al.* 2003), which was seen as slight trend in total T4 with the physiological E2B dose and the high doses of all three test compounds. This parallel increase might indicate an estrogenic effect of all compounds on serum proteins, that became detectable with the larger fraction of circulating TT4.

E2 is frequently reported to promote thyroid carcinogenesis in rat models (Mori *et al.* 1990; Takagi *et al.* 2002; Thiruvengadam *et al.* 2003). Dose-dependent difference in response of the HPT axis to E2 was shown, i.e. TSH increase in combination with normal thyroid hormone concentrations in hyperestrogenized rats (Lisboa *et al.* 1997), which was found in the presented experiment as well.

GEN is discussed for its possible goitrogenic potential because of strong thyroid peroxidase (TPO) inhibition activity. Contrastingly a human trial suggested thyroid cancer prevention due to dietary soy (Doerge *et al.* 2002b; Horn-Ross *et al.* 2002).

It is questionable, if the parallel effects of E2B low and GEN high on serum hormones after long term treatment also correspond with respect to the underlying mode of action. Under any treatment the thyroid was histologically unaffected concerning follicle condition, but their impact on relative thyroid weight differed, where E2B showed an increasing trend, as well as their thyroid gene expression profile, with enhanced mRNA transcription of IGF1 and ER $\alpha$  genes under E2B low.

The overall decrease of T3 and T4 through a single dose of RES is compensated in the long term by elevated total levels, which leaves the animals with free levels that equal CON ovx and intact animals. While RES did not exert any alteration in gene expression of thyroid IGF1, ER $\alpha$  or ER $\beta$ , nor influence relative thyroid weight and histology, it has inhibiting potential on TPO (Schmutzler *et al.* 2004). But its effects cannot be entirely attributed to that activity.

8PN influence on the thyroid axis is not studied so far. Concerning the hormonal status and thyroid gene expression the HPT-axis seemed not as susceptible to the estrogenic potency of 8PN as the HPG-axis. The estrogenic effect with elevated TSH in vitro and also after the single dose application in vivo was lost during long term treatment, and the animals seemed to maintain an euthyroid status with elevated fractions of bound hormones. Though follicle morphology appeared unaltered in the histological assessment, further investigation of the endocrine activity of 8PN in the thyroid is necessary to explain the significant increase of relative organ weight. Without displaying effects on ER $\alpha$  and ER $\beta$  transcription in thyroid nor on pituitary TSH release under chronic conditions, 8PN might elicit an estrogenic stimulus sufficient to increase thyrocyte proliferation.

### 4.3 Multiorgan effects of long term exposure

#### 4.3.1 Reproductive organs

Though the thyroid axis is closely related to reproductive health (Doufas *et al.* 2000; Cooke *et al.* 2004; Jahnke *et al.* 2004), mainly estrogenic influences on female reproductive organs can be detected. This is due to the fact that the organs are permanently exposed to constant thyroid hormone levels, while they are heavily modulated by E2 and progesterone during cycles causing very distinct features.

##### **Uterus**

In gross morphology both doses of E2B increased uterus wet weight approximately 6 fold in comparison to ovx controls was observed. The administration of GEN high raised the weight of uteri about 2 fold, and 8PN high about 3 fold, while RES treatment did not influence the uterus weight at all. The stimulation of the complement protein C3 gene expression, a highly sensitive marker for uterotrophic effects of estrogens, mirrored this finding very well.

Measuring thickness of myometrial and endometrial layers is a standard parameter for estrogen induced proliferation. Since estrogenic proliferative effects could be assumed by the finding of the pronounced weight changes, the fine details of endometrial epithelium and glands were of more interest in order to distinguish even subtle influences of the test substances.

The known morphological changes induced by E2 in uterus, though of varying extent, were found under any treatment except in the control group of ovariectomized animals. First indicators are changes in the stromal layer beneath the epithelium, whose lamina propria cells adopt spindle shape and polarity. This feature was seen throughout all groups, in some animals of GEN low, both RES doses and 8PN low, and in all animals of 8PN high, GEN high and both E2B.

The endometrial epithelium and glands displayed variable responses to the test substances. The effects on cell renewing (cell death and mitotic figures) were mainly seen in the E2B low group, which represented maximal proestrus E2 serum levels. The hyperestrogenized animals of E2B high developed hypertrophic and hyperplastic endometrium epithelium and glands, multilayered epithelium and squamous metaplasia and also pyometra, which is the most severe feature induced by E2.

GEN high induced epithelial proliferation reflected in detectable mitotic activity.

8PN high not only exerted pathological findings such as hypertrophy and hyperplasia of epithelial structures and multilayered endometrial epithelium, but also a novel lesion, that might be described as polypoid structures with intraepithelial cysts.

The appearance of epithelial effects in E2B groups and the 8PN high group seemed to be associated with activation of the uterine IGF1 gene, while the slight proliferative effect of GEN high was not. The stimulation of ER $\alpha$  gene expression did not show any clear trend, while ER $\beta$  expression was slightly downregulated in the groups that had increased uterus weight, i.e. E2B, GEN high and 8PN high. This corresponds with the theory that estrogenic downregulation of ER $\beta$ , which mediates effects of differentiation, would enhance ER $\alpha$ -regulated proliferation.

PR gene expression in uterus is enhanced after exposure to E2 (Faber *et al.* 1976). In an experiment with 6 treatment days it showed rapid time- and dose-dependent response and decline after a 24h peak (Kraus *et al.* 1993). PR gene expression was markedly activated to values of intact animals by the supraphysiological E2 levels of E2B high, that may provide the extra stimulus that is lost in the long term at the physiological E2 level. An elevation of PR transcripts was also detectable in RES high and both 8PN groups. It remains to be clarified, if these findings are true or chance results. Under a three day regimen the estrogen induction of PR transcripts was antagonized by progesterone (Kraus *et al.* 1993). In binding studies no PR activity was detected for 8PN (Milligan *et al.* 2000), but for RES an interaction with PR was reported in human mammary cancer cell lines (Damianaki *et al.* 2000).

The morphological changes induced by GEN and RES in uterus seem to be estrogenic and were increasing from RES groups and GEN low leading to GEN high, but not reaching E2B responses with mild pathological findings and clear proliferative effects of E2B low and severe pathological effects, massive proliferation and preneoplastic signs with E2B high. 8PN might have a different mechanism of action rather than only mimicking E2 action in uterus. While only reaching approximately half of the uterine weight induced by any E2B dose, pathological findings comparable to E2B high were found, plus the different microscopic feature (polypoid structures) only appearing in 8PN high.

### **Vagina**

While control animals had an atrophic epithelium, both E2B treatments increased epithelial number of layers and induced cornification in all samples with an apparent stronger effect of E2B high.

GEN treated animals showed different changes than E2B treated. GEN high induced epithelium growth almost without cornification (only seen in one animal treated with GEN high dose), and under both GEN doses cytoplasmic vacuolization was seen, clear cut effects in the GEN high and beginning effects in the GEN low group. The influence of GEN

on vaginal epithelium seems to be not solely estrogenic action because these morphological findings (cytoplasmic vacuolization of vaginal epithelium with increased epithelium layers) resemble a combined estrogens/progestins stimulation (Haschek *et al.* 2001). In fact, it was reported that in competitive radioligand binding assays GEN shows an affinity to PR within the same range of its affinity to ER $\alpha$  and ER $\beta$  (Beck *et al.* 2003). 8PN also exerted different morphological changes than E2B. 8PN high increased number of layers comparable to E2B high. Again, cytoplasmic vacuolization was detected incipient under 8PN low and clear under 8PN high. Also cornification was never seen in 8PN treated animals, even when the vaginal epithelium adopted a typical squamous multilayered aspect.

In the vaginal tissue the differences from pure estrogenic stimulation were evident. GEN and 8PN induced histological changes, that were not seen under any E2B treatment. Similar features are described in literature to be induced by an administration of high doses estradiol combined with progesterone to ovx animals. The results might indicate a mechanism involving PR activity of GEN and 8PN in vaginal epithelium, that was previously shown for GEN, but not for 8PN (Milligan *et al.* 2000).

#### 4.3.2 Liver

The weight of liver being a metabolic organ may well be correlated with body weight. The relative liver weights were only increased by E2B treatment, were unchanged by the test compounds and only slightly higher in intact animals than in controls, while the E2 regulated genes IGF1 and ER $\alpha$  were not significantly effected even in E2B and intact groups.

Both thyroid hormones and E2 again exert important effects, though this time on metabolic function. The liver is not only important to render T3 available to the system by conversion of T4 with 5'-DI type I, but also for lipid metabolism, which is strongly influenced by E2. After decrease of endogenous E2 in postmenopausal women cholesterol increases the risk of coronary artery disease and atherosclerotic changes in combination with increased levels of LDL.

The lowering effect of E2 on cholesterol and LDL was well demonstrated in intact and E2B treated animals, where in the hyperestrogenized E2B group the decline was stronger than in the intact group. On the other hand only this group showed extremely elevated triglycerides. Since lipid metabolism is differently regulated in the rat the decrease of HDL is not an irritating finding. For soy and GEN decrease of cholesterol and LDL has been claimed in vitro and in vivo (Anthony *et al.* 1996; Alexandersen *et al.* 2001; Borradaile *et al.* 2002), which was not found in any dose of GEN after 3 months treatment. Yet positive

influence of soy could not be proved in human trials with healthy nor slightly hypercholesterolemic volunteers (Hodgson *et al.* 1998; Dewell *et al.* 2002).

The health claim authorized in 1999 by the American Food and Drug administration concerning lowering high cholesterol levels and cardiac risk is coupled to the daily intake of 25g of soy protein, not of the soy isoflavones.

([www.fda.gov/fdac/features/2000/300\\_soy.html](http://www.fda.gov/fdac/features/2000/300_soy.html))

Activity of RES in this context is mainly attributed to its antioxidant properties by preventing oxidation of LDL, which is supposed to be an important factor of atherogenesis (Fremont 2000). In rats it was reported to antagonize the positive effects of E2 on cholesterol (Turner *et al.* 1999). Like in GEN treated animals no decline of cholesterol or LDL was observable in RES treated groups.

But 8PN caused these responses in the high dose, most likely due to its much higher estrogenic potency, less pronounced for cholesterol but well for LDL. Nothing specific is yet published concerning 8PN and lipid metabolism, but it was shown in vitro that the prenylation of flavonoids is improving the inhibition of lipid peroxidation (Rodriguez *et al.* 2001).

In this context it has to be noted that lower body weights than in ovx animals were not only lower in the groups showing effect on lipid metabolism, namely intact, both E2B and 8PN high, but also in GEN high. A fact that corresponded with decreased food intake in these groups and markedly lower levels of leptin, which is released by the fat tissue and controls appetite and also the thyroid axis (Legradi *et al.* 1997).

### 4.3.3 Bone

In the bone the balance of gonadal and thyroid axis and the counteraction of E2 and T3 are extremely important. T3 is responsible for linear growth and maintenance of bone mass (Bassett *et al.* 2003), while E2 maintains bone density.

In ovariectomized-thyroidectomized rats it was shown that the presence of both is required by each hormone to exert its own effect (DiPippo *et al.* 1995). Osteoporosis starts with diminishing E2 levels in the peri- and postmenopausal years (Lindsay 1998), but is also induced by hyperthyroidism, which one quarter of postmenopausal women develop (Schindler 2003).

Surrogate parameters of bone metabolism are the osteoblast product osteocalcin and the osteoblast product RatLaps. The stable n-terminal fragments of osteocalcin, which are bone  $\gamma$ - carboxyglutaminic acid protein fragments, can be measured in blood (Young



2003). RatLaps are products of bone specific collagen 1 $\alpha$ 1, i.e. fragments of C-terminal telopeptides with  $\beta$ -asparaginic acid ( $\beta$ -CTX) (Rosenquist *et al.* 1998).

The thyroid hormone calcitonin regulates plasma calcium concentration by inhibition of osteoclast activity, resulting in decreased mobilization from bone (Zaidi *et al.* 1993a; Zaidi *et al.* 1993b), but its secretion is also regulated by estrogens (Filipovic *et al.* 2003).

The main parameter for E2 activity is trabecular density, where estrogenic effects are well detected. Since bone mass is T3 dependent, total mass (including corticalis and spongiosa) was also evaluated. Resistance of the bone and consequently the strength strain index (SSI) may be impaired by hyperthyroidism resulting in a higher fracture rate, which can be compensated by E2.

Trabecular density was affected strongest by ovariectomy with a drop of 50% in comparison to intact animals or E2B low groups, while total mass and SSI lay only 10% lower.

Both E2B doses maintained bone density quite well, although they did not reach values of intact animals. Total mass and SSI were positively affected only by E2B low, comparably to the intact animals. The lower calcitonin levels in these two groups might indicate regulation within the thyroid gland, where ER $\alpha$  and ER $\beta$  were upregulated.

E2B high resulted in total mass of ovx level and even a significant decrease of SSI, which might be caused by a decline of osteoblast activity suggested by the parallel drop of osteocalcin in this group. Surprisingly E2B low was the only group where RatLaps were markedly increased, which contradicted previous findings, when E2 reduced osteoclast activity though at higher E2 levels (Seidlova-Wuttke *et al.* 2003b). Maybe that effect depends on circulating E2 levels and/or is due to the lowered free T3 levels specifically detected under this treatment. Another possibility is the declining tendency of TSH under E2B low, which was reported to inhibit osteoclasts (Abe *et al.* 2003).

Numerous studies on soy in vitro, in vivo and human observational/dietary intervention show bone-sparing effects in the long term without clear evidence of the underlying mechanism (Setchell *et al.* 2003). For GEN alone no human data are available so far. In mice a dose difference between bone and uterus was claimed (Ishimi *et al.* 2000). GEN as single substance also proved to increase trabecular density, indicating estrogenic mechanisms, without altering total mass or SSI. Only the high dose affected bone, thus a difference of activity in bone and uterus is not supported by the present data because already the low dose already induced slight changes in uterus.

Concerning RES not much is published about effects on bone. It decreased calcium content in bone tissue culture (Yamaguchi *et al.* 2001) and did not influence cortical bone histomorphometry in growing rats (Turner *et al.* 1999). The only finding potentially important for the bone was a significant decrease of calcitonin levels.

The first in vivo publication on 8PN already investigated bone effects, which was logical considering its extraordinary potency for a phytoestrogen (Miyamoto *et al.* 1998). The reported suppression of ovx induced bone loss is substantiated by the considerable increase of trabecular density under 8PN high measured by qCT. Interestingly, the effect was not stronger than under GEN high, which has much lesser binding affinity than 8PN to ER $\alpha$ , that in bone is present in higher concentrations than ER $\beta$ .

## 4.4 Conclusions

### **Gonadal and thyroid axes**

Taken together, results of the in vitro test and the single dose administration in vivo were not generally transferable to any long term treatment effects. Nevertheless cell culture and single dose effects helped in part to explain adaptation processes in the long term.

Effects in the gonadal axis persisted throughout all experiments with 8PN and were lost during chronic treatment with GEN, where all doses were lower than in the single dose treatment.

All substances affected the thyroid axis in vivo. Under chronic exposure the multiple regulating mechanisms achieved a new, maybe slightly skewed, equilibrium.

Mechanisms of action of the test compounds cannot be easily explained by classical feedback effects, but display a complex mixture of variables specific for each substance.

### **Multiorganic effects of long term exposure**

The 3 months treatment of ovx female rats illustrated the complexity of mechanism of action, but allowed good assessment of the effect scenario. Since the substances achieved reasonable serum levels in the animals, that may be reached in humans as well, these results provide useful information for the judgement of risks and benefits of human exposure.

### **Genistein**

GEN products to alleviate menopausal symptoms should be handled with care. Favorable CNS effects could not be seen after long term treatment. Effective total serum levels of at least 25µM are required, as shown in the single dose treatment.

The alterations detected in uterus and vagina even under a low dosage comparable to those achievable by a weak nutritional supplement, call for additional attention.

Thus, the potential bone sparing effects achieved by a dose common in cancer prevention preparations have to be weighed against unfavorable changes within the reproductive organs and a modulated thyroid axis with altered free T3 levels.

### **Resveratrol**

The potential estrogenicity of RES is not evidenced in the used ovx model. Even under a high dose only minor effects on the uterus were detectable after long term treatment.

For human use RES seems not a treatment option for menopausal complaints. On the other hand its antiestrogenic activity in the presence of E2 does not allow to conclude for the effect of RES when used in intact or E2 substituted conditions.

The thyroid effects found in the in vivo experiments are not negligible. They should be considered especially for elevated doses, that can be found in some merchandised preparations.

### **8-Prenylnaringenin**

8PN showed all estrogenic features that could be expected by its strong and preferential binding activity to the ER $\alpha$  concerning estrogen-like effects on LH suppression, lipid metabolism and bone density. Findings in the uterus and the vagina however, require further investigation. They illustrate how overall impression of parameters is differentiated by such details, that might be important aspects of the substance profile.

Whether bust enhancement preparations reach effective concentrations in humans is not known, but the potency of 8PN must not be underestimated.

### **Final conclusion**

The comparison of results obtained by the three different test systems yielded a complex picture. Depending on the experimental design, exposure time and dosage the test compounds caused varying effects.

In all experimental sets GEN, RES and 8PN showed interaction with the regulatory HPG and HPT axes and the related reproductive organs, liver and bone. That might offer the chance for therapeutic treatment or prevention options, but also includes potential risks.

Regarding consumption of preparations containing high doses of GEN, RES or 8PN it is recommendable to reduce the risk of uncontrolled exposure and endocrine disrupting effects. Therefore a clinically approved indication and a regulatory approved status, which includes safety guidance, should be required.

In the context of the underlying European endocrine disruptor research project this study is a contribution to the scientific basis within the European Union to support a coherent strategy in its protection of human health.

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## 7 Fact sheets

## 7.1 Certificates of analysis of test compounds

## 7.1.1 Positive controls

20 NEL135 140530

SIGMA ALDRICH CHEMIE  
GMBH  
GRUNWALDER WEG 30  
D-82041 DEISENHOFEN  
(MUNICH)  
GERMANY

  
**SIGMA-ALDRICH**

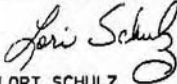
DATE: 10/05/04

-- CERTIFICATE OF ANALYSIS --

PRODUCT NAME: B-ESTRADIOL  
PRODUCT NO: E8875  
LOT NO: 121K1448 FORMULA: C<sub>18</sub>H<sub>24</sub>O<sub>2</sub> CAS NO: 50-28-2

FORMULA WEIGHT: 272.4 STORE AT ROOM TEMPERATURE

TEST	SPECIFICATION	RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 200 MG PLUS 4 ML OF ETHANOL	CONFORMS
PURITY BY HPLC	98% MINIMUM	100%
SHELF LIFE SOP QC-12-006	2 YEARS	JANUARY 2004
QC ACCEPTANCE DATE	JANUARY 2002	

  
LORI SCHULZ  
MANAGER, ANALYTICAL SERVICES  
281/041005#1/CXF5

  
**SIGMA-ALDRICH**

**Certificate of Analysis**

Product Name	β-Estradiol 3-benzoate
Product Number	E8515
Product Brand	SIGMA
CAS Number	50-50-0
Molecular Formula	C <sub>23</sub> H <sub>28</sub> O <sub>3</sub>
Molecular Weight	376.49

TEST	SPECIFICATION	LOT 072K0996 RESULTS
APPEARANCE	WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 50 MG/ML IN CHLOROFORM	CLEAR COLORLESS SOLUTION AT 50 MG/ML IN CHLOROFORM
UV-VIS SPECTRUM	EMM = 18.3 AT LAMBDA MAX 230 NM IN ETHANOL	EMM = 18.3 AT LAMBDA MAX 230 NM IN ETHANOL
PURITY BY HPLC	98.5%	98.5%
QC ACCEPTANCE DATE	JULY 2002	JULY 2002
PRODUCT CROSS REFERENCE INFORMATION	REPLACEMENT FOR ALDRICH #285781	

  
Lori Schulz, Manager  
Analytical Services  
St Louis, Missouri USA

7.1.2 Genistein



Chemical Information Service  
Life Science Products  
Fine Chemicals

Werner-von-Siemensstr. 3  
D-93128 Regenstauf  
Tel 0049 9402 9336 10  
Fax 0049 9402 9336 13  
email sales@chemos-group.com  
http://www.chemos-group.com

CERTIFICATE OF ANALYSIS

Product name: 4, 5, 7-TRIHYDROXYISOFLAVONE Quantity: 30g  
Batch No. SG20030618 Manufacture date: 2003.06.18  
Report date: 2003.06.20 Expiry date: 2005.06.18

Appearance: a white like fine powder  
Specifications:

Item	Limit	Result
Identification	passed test	passed test
Assay (HPLC)	>98%	98.50%
Odor	characteristic	characteristic
Taste	characteristic	characteristic
Mesh size	80mesh	conform
Moisture	<2%	0.60%
Sulphated ash	<2%	0.18%
Heavy metals	<20ppm	conform
As	<3ppm	conform
Solvent residue	<5ppm	conform
Total plate count	<1000cfu/g	conform
Yeast & Mold	<100cfu/g	conform
Salmonella	negative	negative
E. Coli	negative	negative

Conclusion: Passed test according to the Enterprise Standard



Carl Roth GmbH & Co. • 76185 Karlsruhe  
Schwanenstraße 1,5  
Telefon: 0721 • 59 09-0  
Telefax: 0721 • 59 06-149  
E-Mail: info@carlroth.de  
http://www.carlroth.de

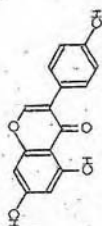
2. Kd. Fr. Binstoffel  
Analysezertifikat  
mit Zusatzdaten.

Datum + Zeichen: 28.07.03 LAU/SCI

Artikelnummer: 3429  
Produkt: Genistein

Charge: 15358965  
Dichte:  
Formel: C<sub>15</sub>H<sub>10</sub>O<sub>5</sub>  
Schmelzpunkt:  
Flammpunkt:  
Reinheit: synth.  
CAS-Nummer: 446-72-0  
Molekulargewicht: 270,23  
Lagertemperatur:  
Siedepunkt:

CAS number [446-72-0]  
Chemical family Flavonoids  
Synonym 4',5,7-Trihydroxyisoflavone  
Molecular formula C<sub>15</sub>H<sub>10</sub>O<sub>5</sub>  
Molecular weight 270,23



ANALYSIS

Appearance	Beige-coloured powder
Melting point	299-302°C, dec
Thin layer chromatography	Silicagel support solvent Toluene / ethylformiate / formic acid (5/4/1, V/V/V) detection U.V.
Absorption maxima	261, (326.5)sh nm in methanol
Assay (HPLC)*	>95% - <i>substant</i>

\* Product not-certified. Assay by an HPLC method.  
The values given on the 'Certificate of Analysis' are the results determined at the time of analysis.

Storage Room temperature.  
Store in a dry and dark place. Place in a desiccator under vacuum for 48 hours before use.

7.1.3 Resveratrol



SIGMA ALDRICH CHEMIE  
 GMBH  
 GRUNWALDER WEG 30  
 D-82041 DEISENHOFEN  
 (MUNICH)  
 GERMANY

DATE: 10/05/04

-- CERTIFICATE OF ANALYSIS --

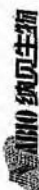
PRODUCT NAME: RESVERATROL  
 PRODUCT NO: R5010  
 LOT NO: 111K5206  
 FORMULA: C<sub>14</sub>H<sub>12</sub>O<sub>3</sub>  
 FORMULA WEIGHT: 228.2  
 STORE IN A FREEZER

TEST	SPECIFICATION	RESULTS
APPEARANCE	OFF-WHITE POWDER	CONFORMS
SOLUBILITY	CLEAR FAINT YELLOW SOLUTION AT 50 MG/ML OF ACETONE	CONFORMS
ELEMENTAL ANALYSIS	73.0 TO 74.3% CARBON	73.4%
IR SPECTRUM	CONSISTENT WITH STRUCTURE	CONFORMS
PURITY BY GAS CHROMATOGRAPHY	GREATER THAN 99%	100%
PURITY BY THIN LAYER CHROMATOGRAPHY	GREATER THAN 99%	100%

QC ACCEPTANCE DATE DECEMBER 2001

*Lori Schulz*

LORI SCHULZ  
 MANAGER, ANALYTICAL SERVICES  
 270/04-1005#1/CXF5



Nabio Biotechnology Co., Ltd

CERTIFICATE OF ANALYSIS

Product Name: Resveratrol  
 Batch Quantity: 1.0kg  
 Analysis Date: June 21, 2003

Batch No.: 20030721  
 Manufacture Date: June 21, 2003  
 Certificate Date: June 22, 2003

IDENTIFICATION	STANDARDS	RESULTS
Appearance Taste Color Resveratrol Solubility Heavy metals(mg/kg) Loss on drying	Drying fine powder Characteristic Pale-Yellow or White 99%Min Resveratrol Solubility in Alcohol ≤10 ≤2.0%	Conform Conform Conform 99.22% Conform 8.44 0.5%
Microbiological		
Total plate count(ctu/g) Mold(ctu/g) Yeast(ctu/g) E.Coli(MPN/100g)	≤1000 ≤25 Negative Negative	Conform ≤5 Non-detective Non-detective

Conclusion:

Storage: Conform with specification  
 Store in cool, dry and dark place

Shelf life: 2years when properly stored

Li Xiaoping

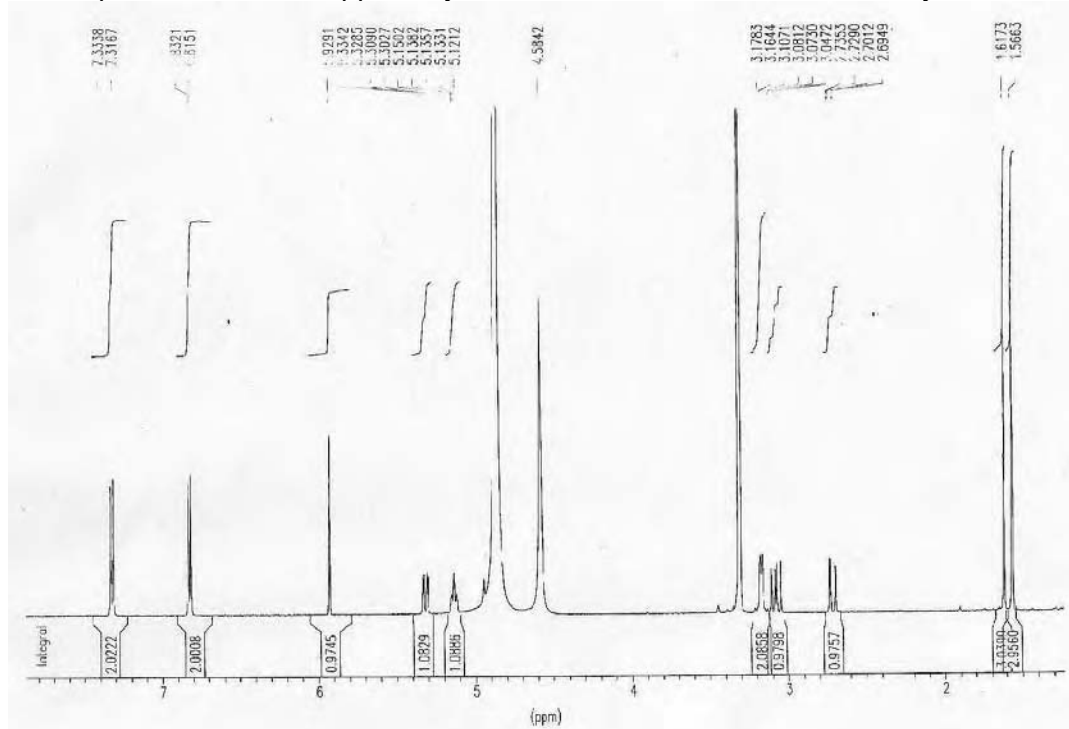
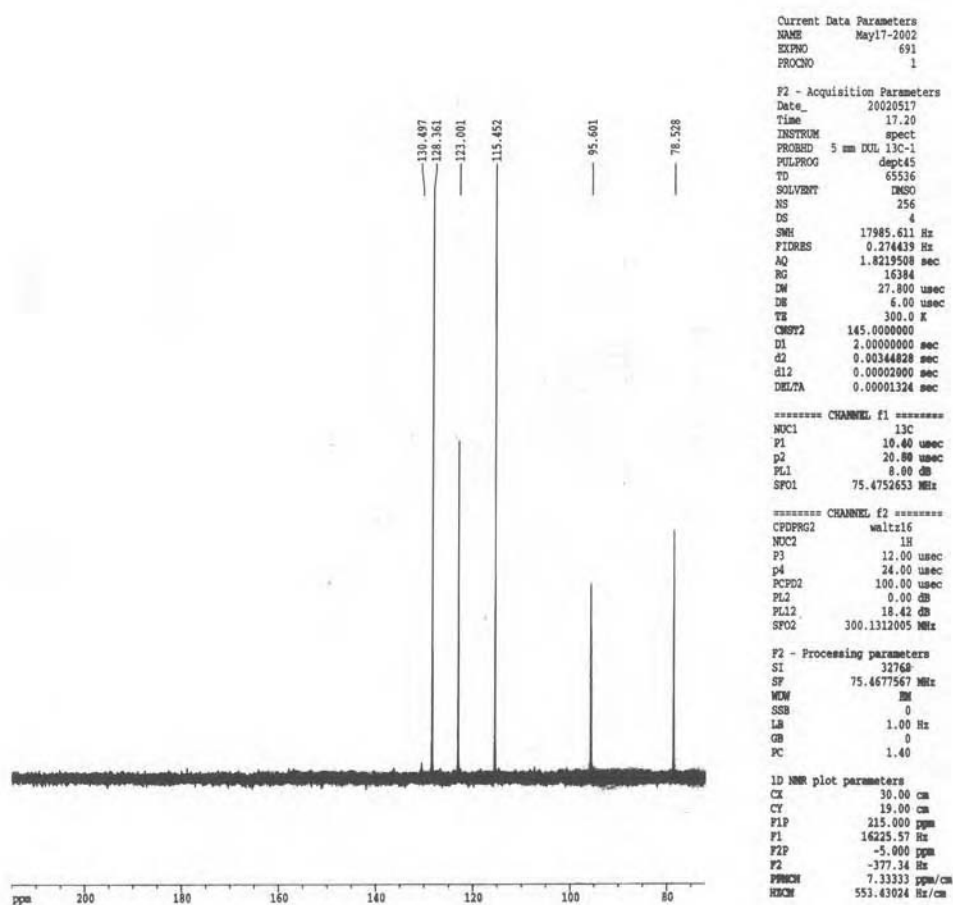
Quality Assurance Officer



Chen Jing  
 Connector

## 7.1.4 8-Prenylaringenin

NMR spectrum of 8PN supplied by Prof. H. Becker, Saarland University, Saarbrücken:

<sup>13</sup>C-DEPT spectrum of 8PN in DMSO, 8PN supplied by Schering AG, Berlin:

## 7.2 Composition of soyfree rat diet

**ssniff® SM R/M, phytöstrogenarm (Soja-frei)**  
**S8585-S010** *Ratten*
**Nährstoffgehalt**

<b>Rohnährstoffe</b>	<b>[%]</b>	<b>Mineralstoffe</b>	<b>[%]</b>
Trockensubstanz	88,0	Calcium	1,00
Rohprotein (N x 6,25)	19,1	Phosphor	0,70
Rohfett	2,2	Natrium	0,20
Rohfaser	4,1	Magnesium	0,17
Rohasche	5,8	Kalium	0,70
Stärke	40,0		
Zucker	3,1		

**Energie** [MJ/kg]

Bruttoenergie, GE	16,2
Umsetzbare Energie, ME	12,9

**Verteilung der ME****Fettsäuren** [% im Futter]

C 16:0	0,33
C 18:0	0,03
C 18:1 n9	0,32
C 18:2 n6	1,18
C 18:3 n3	0,17

**Vitamine** [pro kg]

Vitamin A	15.000 IE
Vitamin D <sub>3</sub>	1.000 IE
Vitamin E	110 mg
Vitamin K	5 mg
Vitamin B <sub>1</sub> (Thiamin)	19 mg
Vitamin B <sub>2</sub> (Riboflavin)	21 mg
Vitamin B <sub>6</sub> (Pyridoxin)	19 mg
Vitamin B <sub>12</sub> (Cobalamin)	100 µg
Biotin	450 µg
Pantothensäure	35 mg
Folsäure	6 mg
Nicotinsäure	105 mg
Cholinchlorid	2.450 mg

**Spurenelemente** [pro kg]

Eisen	145 mg
Mangan	80 mg
Zink	100 mg
Kupfer	14 mg
Iod	2,1 mg
Selen	0,4 mg
Cobalt	2,1 mg

**Aminosäuren**

Lysin	1,04 %
Methionin	0,35 %
Met+Cys	0,70 %
Threonin	0,78 %
Tryptophan	0,25 %
Arginin	0,95 %
Histidin	0,42 %
Valin	1,03 %
Isoleucin	0,85 %
Leucin	1,48 %
Phenylalanin	0,97 %
Phe+Tyr	1,74 %
Glycin	0,88 %
Glutaminsäure	3,75 %

**Zusammensetzung:** Getreide, Pflanzliche Nebenerzeugnisse, Pflanzliche Eiweißextrakte, Pflanzliche Fette, Mineralstoffe, Vitamine und Spurenelemente.

### 7.3 Cell culture materials

Spinner and Dulbecco's Modified Eagle Medium:	Gibco
SMEM	cat.# 21385-026
DMEM	cat.# 41965-039
DMEM w/o phenolred	cat.# 11880-028
Phosphate buffered saline (w/o Ca and Mg, 1:10 dilution)	
Na-pyruvate (100mM)	
Glutamine (200mM)	
Penicillin/Streptomycin (10T Units/ml PenG, 10T µg/ml Streptomycin)	
Horse serum	
Fetal calf serum with and w/o steroids	
Bovine serum albumin 7,5%	
Hepes buffer (1M in 0,85% NaCl)	Biowhittaker
MTT (Dimethylthiazol-tetrazolium bromide)	Sigma
Glutardialdehyde (25% aqueous solution)	

### 7.4 HPLC materials

HPLC Pump K-501	Knauer
Dynamic mixing chamber	
Interface box	
Chromgate (Integrator and analysis program)	
851-AS Intelligent sampler	Jasco
LC-95 UV/Visible Spectrophotometer Detector	Perkin-Elmer
RF-10A XL Fluorescence Detector	Shimadzu

#### For food analysis:

Column	NC-04 250x4,0mm Hypersil-OD1 5,0µm P/N 2504 F180HY050	Bischoff
Precolumn	10µm C18 Shandon ODSiiRP	Shandon

#### For serum analysis:

Column	NC 250 x 4,6mm Hypersil-ODS 5,0µm P/N 2546 1805, S/N 2711C30	Bischoff
Precolumn	Guard 7,5 x 4,6mm SS Jour-Guard RP/C18 5µm P/N 69080, S/N 2110362.1	Jasco

## 8 Abbreviations

Generally SI-units (Système International d'Unités) were used as they are defined and published by the Bureau International des Poids et Mesures.

4MU	4-methyl-umbelliferone
5'-DI	5'-iodothyronine deiodinase
8PN	8-prenylnaringenin
bp	base pairs
BSA	bovine serum albumin
bw	body weight
C3	complement 3
cDNA	complementary DNA
CT	computer tomography
Ct	threshold cycle
DEPC	diethylpyrocarbonate
dNTP	2'-deoxyribonucleoside 5'-triphosphate
DTT	dithiothreitol
E2	17beta estradiol
E2B	17beta estradiol benzoate
ELISA	enzyme linked immunosorbent assay
ER ( $\alpha,\beta$ )	estrogen receptor (subtypes $\alpha+\beta$ )
ERE	estrogen response element
EURISKED	European Union RISK assessment of Endocrine Disruptors research consortium
F0	filial generation 0 (first completely soyfree)
FAM	6-carboxy-fluorescein
FCS	fetal calf serum
FI	fluorescence
FRET	fluorescence resonance energy transfer
FSH	follicle stimulating hormone
FT3/FT4	free T3/T4 = not bound to serum carrier proteins
GEN	genistein
GnRH	gonadotropin releasing hormone
HDL	high density lipoprotein
HE	hematoxylin eosin staining
HEPES	2-[4-(hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid
HPA	hypothalamo-pituitary-adrenal
HPG	hypothalamo-pituitary-gonadal
HPG-axis	gonadal axis
HPLC	high performance liquid chromatography
HPT	hypothalamo-pituitary-thyroid
HPT-axis	thyroid axis
HRT	hormone replacement therapy
i.p.	intraperitoneal
ICI	steroidal antiestrogen ICI 182,780 (=fulvestrant, Faslodex)
IGF1	insulin like growth factor
LDL	low density lipoprotein
LH ( $\beta$ )	luteinizing hormone (specific $\beta$ subunit)
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
mRNA	messenger ribonucleic acid
MTT	3,(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl-tetrazolium-bromide
n.d.	not determined



---

NIEHS	National Institute of Environmental Health Sciences
NIH	National Institute of Health
NMR	nuclear magnetic resonance
OD	optical density
OECD	Organization for Economic Co-operation and Development
ovx	ovariectomized
p.o.	per os
PAP	Papanicolaou stain
PAS	periodic acid Schiff stain
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PR	progesterone receptor
R2	correlation coefficient
RES	trans-resveratrol
RIA	radio immuno assay
RNase	ribonuclease
RP	reversed phase
RT	room temperature
RT-PCR	reverse transcriptase PCR
SD	standard deviation
SEM	standard error of the mean
SERM	selective estrogen receptor modulator
SSI	strength strain index
STD	standard
T3	triiodothyronine
T4	thyroxine = tetraiodothyronine
TAMRA	6-carboxy-tetramethyl-rhodamine
taq	thermophilus aquaticus
TPO	thyroid peroxidase
TRH	thyrotropin releasing hormone
tris	tris-(hydroxymethyl)aminomethane
TSH ( $\beta$ )	thyroid stimulating hormone = thyrotropin (specific $\beta$ subunit)
TT3/TT4	total T3/T4 = unbound+bound to serum carrier proteins
UV	ultraviolet light

## **Statement of Originality**

I declare that this thesis has not been submitted in any form for another degree or diploma at any university. Results discussed in this thesis are my own work, and information derived from the literature or unpublished work of others has been acknowledged in the text and a list of references provided.

## **Erklärung**

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel von mir eingesetzt worden sind. Des weiteren versichere ich, dass die vorliegende Dissertation weder ganz noch zum Teil bei einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegen hat.

Göttingen, 15.12.2004

# Curriculum Vitae

## PERSONAL DATA

*Name* Julie Christoffel  
*Birth* 14th of April 1972 in Würzburg, Germany  
*Nationality* German

## PROFESSIONAL DATA

### *PhD THESIS*

2002/11 - 2004/12 Experimental work in the Dept. of Clin. and Exp. Endocrinology, Prof. Dr. W. Wuttke, Division of Obstetrics and Gynecology, University of Göttingen, Germany

### *OCCUPATIONAL ACTIVITIES*

1997/8 - 2002/10 Employment as approved pharmacist in the public pharmacy Apotheke Königswiesen, S. Yersin-Adler, Regensburg, Germany

### *ADDITIONAL QUALIFICATIONS*

1998/11 - 2002/9 Certificate as „Specialized Pharmacist for the public pharmacy“ (Fachapothekerin für Offizin-Pharmazie) Certificate of the Chamber of German Pharmacists (Bayerische Landesapothekerkammer)

1998/11 – 2000/10 Certificate as „MBA for Pharmacy“ (Praktische Betriebswirtin für die Pharmazie), concurrent postgraduate studies at the Academy of German Pharmacists (Wirtschaftsakademie Deutscher Apotheker), Frankfurt, in collaboration with the University of Bayreuth

### *SCIENTIFIC EDUCATION*

1997/7 Approbation as Approved Pharmacist

1996/11 – 1997/4 Practical work in the public pharmacy Apotheke Königswiesen, S. Yersin-Adler, Regensburg, Germany

1996/5 – 1996/10 Practical work in the Department of Dermatology,  
Prof. H. I. Maibach, M.D., University of California, San Francisco,  
USA

1992/5 – 1996/4 Studies of pharmacy at the University of Regensburg, Germany

1991/10 – 1992/3 Studies of chemistry at the University of Regensburg, Germany

1991/7 General qualification for university entrance (Abitur) at the Domgymnasium, Freising, Germany