Regulation of Porcine Hepatic Cytochrome P450 by Chicory Root – Implication for Boar Taint

PhD Thesis

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Preface

The present PhD thesis provides novel knowledge of the impact of dietary compounds on liver metabolism in pigs. The project was initiated to suggest a solution to reduce the occurrence of tainted meat from boars. The occurrence of boar taint is partly related to hepatic Phase I and II metabolism. Today's answer to the boar taint problem is surgical castration of male piglets without anaesthetics, before the age of 7 days. Due to increasing consumer awareness and political pressure, within EU, this practise will end latest 2018. Several alternatives to surgical castration have been suggested, among them diets enriched in components with bioactive properties.

The presented work has been conducted at Department of Food Science (Foulum), Faculty of Science and Technology, Aarhus University, from April 2009 until July 2012. Likewise, work has been done during a stay at Galia Zamaratskaia's lab, Department of Food Science, SLU BioCentrum, Uppsala, Sweden, in 2010.

During the project, additional work has been done on the regulation of hepatic Phase I and II enzymes by steroids of testicular origin. This work (see "*List of supporting publications*") is not included in the thesis, but contributes to the knowledge about boar taint.

Acknowledgement

My main supervisor Bo Ekstrand, Department of Food Science (Foulum), Aarhus University, is greatly acknowledged for being more than a mentor for me. You have always taken an interest in me and taken your time to discuss both scientific and non-scientific matters with me. Together we have learned a lot about cytochrome P450, detoxification, boar taint and all the exciting things surrounding these topics. During the project I was fortunate to have Galia Zamaratskaia, Department of Food Science, SLU BioCentrum, Uppsala, Sweden, as my co-supervisor. You made a significant difference for the project and me. Thank you for letting me visit your lab and sharing all you knowledge with me, for sharing your ideas and offering me the opportunity to be part of them. Bo and Galia, I could not have asked for better support – I could not have done this work without the both of you.

The presented work could not have been done without the valuable contributions from a number of great people. Thanks to Carl Brunius, Department of Food Science, SLU BioCentrum, Uppsala, Sweden, for fruitful discussions of the boar taint problem, as well as helping with the analyses of the boar taint compounds. Hopefully, we can continue our discussions in the future. Christina Lindgaard Jensen, Department of Food Science (Foulum), Aarhus University, is

acknowledged, for conducting numerous attempts during the development of the protocol for isolation and culturing of primary hepatocytes. It was hard work, but we made it at last. Thanks to Jette Feveile Young, Department of Food Science (Foulum), Aarhus University, for teaching and discussing cell work and RT-PCR with me. Bente Andersen and Jens Askov Jensen, Department of Food Science (Foulum), Aarhus University, are acknowledged for their help in the lab and during sampling at the slaughterhouse.

Last but not least my beloved family deserves to be acknowledged. Maria you make a difference to me, you mean more to me than words can describe. Thank you for believing in me, your support and all the other stuff that we share. To our children, Lærke and Victor, you are unique – I love you. Maria, Lærke and Victor you make my life complete. To my mom, Lene, and my dad, Jørgen, thanks for your love and support. Also thanks to Erik and Marianne for your support and interest in the project.

The financial support from DLG is greatly acknowledged.

Martin Krøyer Rasmussen

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List of contents

Abstract

Dietary components have been shown to affect the hepatic detoxification system. Hepatic clearance of xenobiotics is performed in two steps, Phase I and II. Phase I is usually carried out by enzymes belonging to the cytochrome P450 (CYP) family, while Phase II is carried out by a more diverse group. Phase I and II enzymes are extensively studied for their importance in drug clearance and in pigs for their impact on the occurrence of boar taint. It is generally believed that sufficient Phase I and II metabolism of the boar taint compounds, skatole and androstenone, diminish the occurrence of tainted meat from sexually mature male pigs. Moreover, dietary chicory root has been shown to decrease the occurrence of boar taint.

The present PhD study was carried out order to investigate the regulation of Phase I and II enzymes by chicory root. Sexually mature male pigs were fed chicory root for the last 16 days before slaughter. They showed increased mRNA expression of CYP1A2, 2A19, 2D25, 2E1, 3A29 as well as 3β hydroxysteroid dehydrogenase (3β-HSD). A simultaneous increase in protein expression of CYP1A2, 2A19 and 3β-HSD as well as increased activities of CYP1A2, 2A and 3A were shown. No effect was shown on the expression of the investigated Phase II enzyme, sulfotransferase 2A1 (SULT2A1). Chicory feeding had no effect on skatole accumulation in fat, while the androstenone concentration found in the group of chicory fed pigs were lower than in the control group. Additionally, chicory feeding had no effect on testosterone, estradiol and insulin-like growth factor 1 (IGF-1) in plasma.

The obtained *in vivo* results of chicory feeding are in contrast to the results obtained *in vitro* using primary porcine hepatocytes. Porcine hepatocytes were isolated from female piglets and treated with a methanolic extract of chicory root for 24 hours. This treatment down regulated the mRNA expression of CYP1A2, 2C33, 2D25 and 3A29. In comparison standard CYP inducers (β-naphthoflavone and dexamethasone) and secondary plant metabolites (artemisinin and scoparone) increased the mRNA expression of the same CYPs.

As the occurrence of boar taint is associated with sexually mature male pigs, a comparison of the constitutive CYP expression and activity between genders was also included. It was shown that female pigs, in comparison to entire male pigs, had greater mRNA expression of CYP1A2, 2A19 and 3A29, while no differences were observed in protein expression. Likewise female pigs had greater CYP1A2, 2A and 2C enzyme activity. The absolute activities found in the chicory fed male pigs were comparable to the activities found in the female pigs.

In conclusion, the results presented in this PhD thesis suggest that chicory feeding can be used to lower the occurrence of boar taint. This is partly based on the finding that chicory feeding increases CYP activity to levels comparable with female pigs, as well as the effects shown on the metabolism of androstenone.

Resumé

Forskellige fødevarer har vist sig at have stor betydning for leverens metaboliske processor. Leverens omsætning af bl.a. giftstoffer sker i to faser, Fase I og II. Fase I katalyseres normalt af enzymer tilhørende cytochrome P450 (CYP) familien, mens Fase II katalyseres af en mere blandet gruppe af enzymer. Fase I og II enzymer er grundigt studeret for deres betydning for omsætningen af lægemidler og i grise for deres betydning for forekomsten af ornelugt. Det er generelt accepteret at tilstrækkelig omsætning af skatol og androstenon i leveren, mindsker tilfældene af kød med ornelugt. Tidligere forsøg har vist at fodring med cikorierod reducerer forekomsten af ornelugt.

Dette Ph.d. projekt blev udført for at studere cikories betydning for reguleringen af Fase I og II enzymer. Kønsmodne hangrise blev fodret med cikorierod de sidste 16 dage optil slagtning. Det blev vist, at de havde øget mRNA ekspression of Fase I enzymerne CYP1A2, 2A19, 2D25, 2E1, 3A29 samt 3β hydroxysteroid dehydrogenase (3β-HSD). Ligeledes havde de øget protein ekspression af CYP1A2, 2A19 og 3β-HSD, samt aktivitet af CYP1A2, 2A og 3A. Der blev ikke fundet nogen effekt af cikorie på det undersøgte Fase II enzym, sulfotransferase 2A1 (SULT2A1). Cikoriefodring havde ingen effekt på skatol ophobningen i fedtvæv, mens androstenon koncentrationen i gruppen af cikoriefodret grise var mindre. Cikoriefodring havde ingen effekt på plasma koncentrationerne af testosteron, østradiol og Insulin-like growth factor 1 (IGF-1).

De fundne *in vivo* resultater var i kontrast til *in vitro* resultaterne opnået ved brug af primære leverceller. Levercellerne var isolerede fra unge hungrise og behandlet i 24 timer med et methanol ekstrakt af cikorierod. Denne behandling nedregulerede mRNA ekspressionen af CYP1A2, 2C33, 2D25 og 3A29. Til sammenligning opreguleredes ekspressionen af de samme CYPer af CYP induktorer (β-naphthoflavone og dexamethasone) og sekundære plante metabolitter (artemisinin og scoparone).

Da forekomsten af ornelugt er associeret med kønsmodne hangrise, er en sammenligning mellem den basale ekspression og aktivitet af CYPerne hos han- og hungrise inkluderet. Det blev vist at hungrise havde større mRNA ekspression af CYP1A2, 2A19 og 3A29, mens der ikke blev fundet nogle forskelle i protein ekspression. Ligeledes havde hungrise større aktivitet af CYP1A2, 2A og 2C. De fundne værdier for CYP aktivitet i cikoriefodret hangrise var sammenlignelige med dem fundet i hungrisene.

Ud fra resultaterne præsenteret i denne Ph.d. afhandling kan det konkluderes at cikorierigt foder kan bruges til at nedbringe forekomsten af ornelugt. Dette er til dels begrundet med fundet af sammenlignelige CYP aktiviteter hos hungrisene og de cikoriefodrede hangrise, samt cikoriefoderets direkte effekt på androstenon omsætningen.

List of included publications

This PhD thesis is based on work presented in the following papers, referred to by their Roman numbers in the text:

I. **Rasmussen, M.K.,** Ekstrand, B., Zamaratskaia, G. (2011). Comparison of cytochrome P450 concentrations and metabolic activities in porcine hepatic microsomes prepared with two different methods. *Toxicology in Vitro 25: 343-346*

II. **Rasmussen, M.K.,** Zamaratskaia, G., Ekstrand, B. (2011). Gender-related differences in cytochrome P450 in porcine liver – implication for activity, expression and inhibition by testicular steroids. *Reproduction in Domestic Animals 46: 616-623*

III. **Rasmussen, M.K.,** Zamaratskaia, G., Ekstrand, B. (2011). *In vivo* effect of dried chicory root (*Cichorium intybus* L.) on xenobiotica metabolizing cytochrome P450 enzymes in porcine liver. *Toxicology Letters 200: 88-91*

IV. **Rasmussen, M.K.,** Brunius, C., Zamaratskaia, G., Ekstrand, B. (2012). Feeding dried chicory root to pigs decreases androstenone accumulation in fat by increasing hepatic 3β hydroxysteroid dehydrogenase expression. *Journal of Steroid Biochemistry and Molecular Biology 130: 90-95*

V. **Rasmussen, M.K.,** Zamaratskaia, G., Andersen, B., Ekstrand, B. (2012). Dried chicory root modifies the activity and expression of hepatic CYP3A but not 2C – effect of *in vitro* and *in vivo* exposure. *Submitted*

VI. **Rasmussen, M.K.,** Jensen, C.L., Ekstrand, B. (2012). Chicory root (*Cichorium intybus* L.) extract down-regulates cytochrome P450 mRNA expression in porcine hepatocytes. *Submitted*

Contributions

The contributions by Martin Krøyer Rasmussen to the papers included in the thesis are as follows:

I. Participated in planning of the study. Prepared microsomes and conducted Western blotting, as well as data analysis and interpretation. Participated in preparation of the manuscript.

II. Participated in planning of the study. Collected and prepared samples for analysis. Carried out all PCR and Western blotting as well as assisted in measuring enzyme activity. Analysed and interpreted data. Responsible for preparation of the manuscript.

III. Participated in planning of the study. Collected and prepared samples for analysis. Made all expression analysis and assisted in measuring enzyme activity. Analysed and interpreted data. Responsible for preparation of the manuscript.

IV. Participated in planning of the study. Collected and prepared samples for analysis. Conducted all PCR and Western blotting and assisted in measuring skatole, indole and androstenone. Analysed and interpreted data. Responsible for preparation of the manuscript.

V. Participated in planning of the study. Collected and prepared samples for analysis. Carried out PCR. Participated in analysing and interpretation of the results. Responsible for preparation of the manuscript.

VI. Responsible for planning of the study. Developed the protocol for isolation and culturing of primary hepatocytes. Conducted all experimental work. Analysed and interpreted data. Responsible for preparation of the manuscript.

List of supporting publications

The work presented in these publications was performed during the PhD study to gain further knowledge about regulation of porcine Phase I and II enzymes, in relation to boar taint. These publications are not included in the thesis, and are therefore referred to in the common way.

1. **Rasmussen, M.K.,** Zamaratskaia, G., Ekstrand, B. (2011). *In vitro* cytochrome P450 2E1 and 2A activities in the presence of testicular steroids. *Reproduction in Domestic Animals 46: 149-154.*

2. Zamaratskaia, G., **Rasmussen, M.K.,** Herbin, I., Ekstrand, B., Zlabek, V. (2011). *In vitro* inhibition of porcine cytochrome P450 by 17β-estradiol and 17α-estradiol. *Interdisciplinary Toxicology 4: 78-84.*

3. Brunius, C., **Rasmussen, M.K.,** Lacoutiére, H., Andersson, K., Ekstrand, B., Zamaratskaia, G. (2012). Expression and activities of hepatic cytochrome P450 (CYP1A, CYP2A and CYP2E1) in entire and castrated male pigs. *Animal 6:2: 271-277.*

4. Tomankova, J., **Rasmussen, M.K.,** Andersson, K., Ekstrand, B., Zamaratskaia, G. (2012). Improvac does not modify the expression and activities of the major drug metabolizing enzymes cytochrome P450 3A and 2C in pigs. *Vaccine 30: 3515-3518.*

5. **Rasmussen, M.K.,** Brunius, C., Ekstrand, B., Zamaratskaia, G. (2012). Expression of hepatic 3β-hydroxysteroid dehydrogenase and sulfotransferase 2A1 in entire and castrated male pigs. *Molecular Biology Reports 39: 7927-7932.*

Abbreviations

AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variation
AO	Aldehyde oxidase
Arnt	AhR nuclear translocator
CAR	Constitutive androstane receptor
cDNA	Complementary DNA
CYP	Cytochrome P450
FXR	Farnesoid X receptor
HPLC	High-performance liquid chromatography
HSD	Hydroxysteroid dehydrogenase
IGF-1	Insulin-like growth factor 1
PCR	Polymerase chain reaction
PXR	Pregnane X receptor
RXR	Retinoid X receptor
STAT	Signal transducers and activators of transcription
SULT	Sulfotransferase
UDP	Uridine diphosphate
UGT	Uridine diphosphate glucuronosyltransferase
XR	Xenobiotic receptor

1.0 Introduction

The hepatic metabolism is essential for the elimination of toxic compounds and has been the focus of much research, especially in relation to the development and safety evaluation of new drugs. Likewise, the liver plays a major role for processing of nutrients. Accordingly the liver and its functions are essential for the health status of the individual. Liver function and its metabolic pathways are affected by several factors, including diet. In pigs, the function of the liver is also of importance for the occurrence of boar taint, a phenomenon with great impact on the sensory precipitation of porcine meat.

1.1 The liver

The liver is a central organ in the body and is responsible for a great number of functions, including synthesis of proteins as well as metabolism of amino acids, lipids, carbohydrates and endogenous compounds; and the detoxification of xenobiotic (drugs, antibiotics, etc.).

The liver is situated in the abdomen, just below the diaphragm where it is surrounded by a thin capsule of connective tissue. The porcine liver is divided into three lobes; the median and the right and left lateral lobe (Court et al. 2003; Filipponi et al. 1995). The median lobe is further subdivided into two separate halves, giving the porcine liver an appearance of consisting of four lobes. This anatomic appearance is in contrast to the human liver, which is divided into a right and a left lobe, based on its splitting into two halves by the falciform ligament. However, both the porcine and human liver can be divided into more lobes based on blood supply (Court et al. 2003; Filipponi et al. 1995; Tsung and Geller 2011). For the human liver, the hepatic artery and the portal vein supply the liver with blood, accounting for ~ 25% and ~ 75%, respectively. The liver is drained by the superhepatic inferior vena cava. Via the portal vein, the liver receives all afferent blood from the small and most of the large intestine, as well as the spleen and pancreas (Soto-Gutierrez et al. 2011). Together with a branch of the portal vein and the hepatic artery, the biliary system makes up the portal triad (Figure 1). Three to six portal triads surrounds the functional unit of the liver; the liver lobules. Within the liver lobules the portal vein and hepatic artery branches into small blood vessels that are surrounded by hepatocytes.

The hepatocytes make up approximately 60 % of all cells in the liver, the rest being endothelial cells, Kupffer cells, stellate cells and fibroblasts.

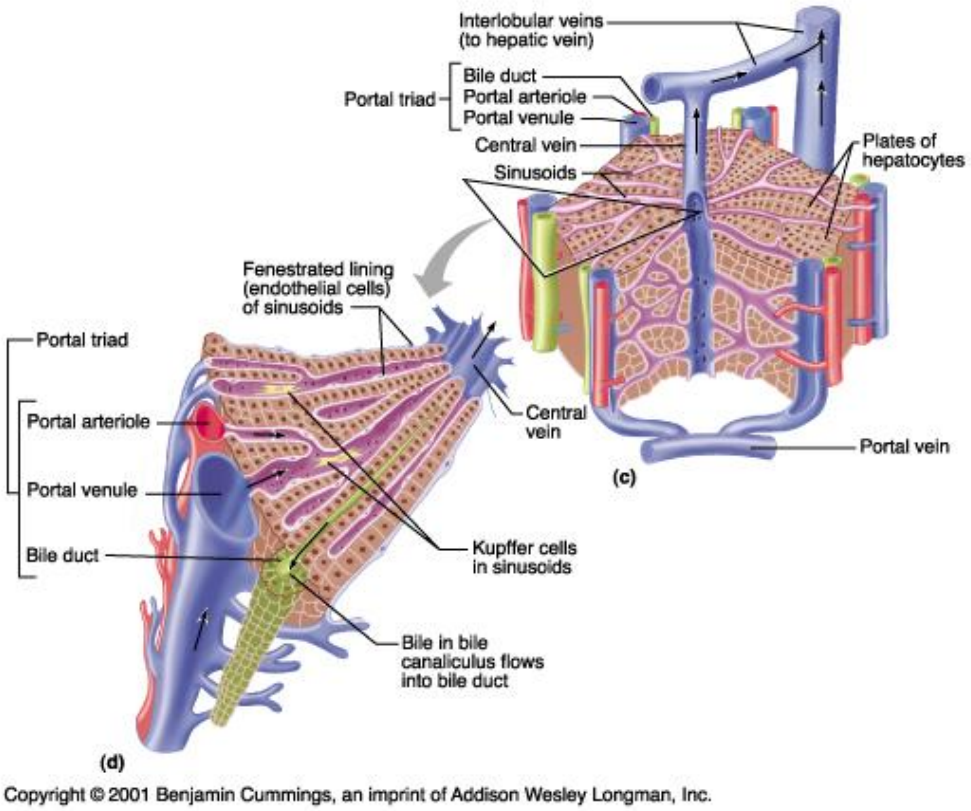

Figure 1. Morphology of the liver. The functional unit of the liver is surrounded by three to six portal triads. Each triad consist of branches of the veins, artery and bile ducts surrounding the hepatocytes. The hepatocytes contain the enzymes responsible for detoxification.

1.2 Hepatic detoxification

Hepatocytes contain the enzymes responsible for the metabolism of xenobiotics. Traditionally, the metabolism of xenobiotics is divided into two phases: Phase I and Phase II. Phase I is usually conducted by enzymes performing hydrolysis, reduction or oxidation, while Phase II enzymes perform conjugations (Parkinson 2001). Thus, Phase I often adds or exposes a functional group, making the compound more hydrophilic, while Phase II, results in an increase in hydrophilicity of the Phase I metabolite, significantly promoting its excretion.

1.2.1 Phase I metabolism

Among the enzymes responsible for the Phase I reactions, the cytochrome P450 monooxygenases (CYP) are the most important. The CYPs are heme-containing enzymes oxidising a wide range of substrates in collaboration with NADPH oxidoreductase and/or cytochrome b_5 (Apte and Krishnamurthy 2011). The overall reaction is as follows: the substrate (RH) is oxidised by incorporation of one oxygen atom to generate the product (ROH), using NADPH as a co-factor.

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

The different isoforms of CYPs are classified according to their amino acid sequence; with isoforms sharing more than 40 % being members of the same family (CYP1, CYP2, CYP3 etc.) and isoforms sharing more than 55 % being members of the same subfamily (CYP1A, CYP1B, CYP2A, CYP2B etc.). The individual isoforms are named by an Arabic number following the designation of the subfamily (CYP1A1, CYP1A2, CYP1B1 etc.).

In the present thesis the focus will be on the porcine versions of the CYPs, including references to the human counterparts when porcine data are scarce or missing. The homology between the human and porcine CYPs is high, ranging between 87 % for human CYP2A6 and porcine CYP2A19 to 62 % for human CYP2C8 and porcine CYP2C33 (Puccinelli et al. 2011). In the porcine liver a number of CYPs are identified, representing the major families CYP1, 2 and 3. Proteomic investigations have shown that the most abundant subfamily is CYP2A (34 %), followed by CYP2D (26%), CYP2C (16 %), CYP3A (14 %) and CYP2E (8 %) (Achour et al. 2011). The most abundant isoforms are CYP2A19 and CYP2D25 making up 60 % of the total amount of CYPs.

Regulation of CYPs

Porcine CYPs are extensively studied for their potential as models for human CYPs (Puccinelli et al. 2011), their involvement in metabolism of veterinary drugs (Antonovic and Martinez 2011; Fink-Gremmels 2008) as well as their importance for boar taint (See *"1.3 Boar Taint"*). The expression of individual CYPs are controlled by ligand binding receptors and has been shown to adapt to the challenges that the liver is presented with. At least three receptors have been shown to regulate CYP expression: aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR; NR1I2) and pregnane X receptor (PXR; NR1I3) (Pascussi et al. 2004; Tompkins and Wallace 2007). Together, these receptors are often referred to as xenobiotic receptors (XR). AhR is located

in the cytosol of the hepatocytes, where it binds to ligands like polycyclic aromatic hydrocarbons and β-naphtoflavone. Upon binding, the receptor-ligand complex translocates to the nucleus, where it associates with AhR nuclear translocator (Arnt). The AhR-Arnt complex binds to the response element of the DNA and turns on the gene transcription (Honkakoski and Negishi 2000; Pascussi et al. 2004). AhR is generally believed to regulate the expression of members of the CYP1A family, e.g. CYP1A2. Both CAR and PXR belong to the family of nuclear receptors. PXR is, like AhR, located in the cytosol where it binds to ligands like rifampicin. Upon binding, it translocates to the nucleus, where it associates with retinoid X receptor (RXR) and initiates gene transcription (Timsit and Negishi 2007). CYP3A is believed to be regulated by PXR. In contrast to AhR and PXR the function of CAR is more complex. Like PXR, CAR associates with RXR and switch on gene transcription, however this can occur in the absence of ligand binding. Compounds like androstanol and androstenol has been identified as inverse agonists, repressing the constitutive activity of CAR (Tolson and Wang 2010). Phenobarbital is a typical ligand for CAR and induces the expression of CYP genes like *CYP2B* (Timsit and Negishi 2007). However, gene regulation is not always straight forward as extensive cross-talk between the XRs does exist as well as between species variation due to difference in the ligand binding domains of the receptors (Pascussi et al. 2004; Pascussi et al. 2008; Tolson and Wang 2010). It is worth noticing that not all CYPs are regulated on the transcriptional level; e.g. CYP2E1 has been suggested to be regulated trough protein stabilization (Tompkins and Wallace 2007).

1.2.2 Phase II metabolism

The Phase II metabolism of xenobiotics often follows Phase I metabolism, making the Phase I metabolite more hydrophilic and thereby enhancing the excretion via the bile and/or urine. Enzymes conducting Phase II metabolism can be grouped into many different superfamilies, based on their conjugation properties (Apte and Krishnamurthy 2011). Glucuronidation, sulfation, glutathione conjugation, acetylation, methylation and amino acid conjugation are the common conjugation reactions conducted by Phase II enzymes, with glucuronidation and sulfation being the major pathways (Parkinson 2001). Glucuronidation is performed by UDP-glucuronosyltransferases (UGT), transferring glucuronic acid to its substrate (Tukey and Strassburg 2000), while enzymes belonging to the sulfotransferases (SULT) adds sulfonate to their substrates (Strott 2002).

In general, Phase II reaction rates are much faster than Phase I, making the latter the rate limiting step in the elimination of xenobiotics (Parkinson 2001).

1.3 Boar taint

Boar taint is an offensive odour released upon heating of meat from some sexually mature male pigs, mainly caused by two compounds: skatole and androstenone. Other compounds like indole, androstenols and *p*-cresol have also been suggested to cause boar taint (Zamaratskaia and Squires 2009), but skatole and androstenone are acknowledge to be the main contributors and will be the focus of this thesis. Liver function and in particular Phase I and II metabolism has been the focus of much research in pigs, due to its implication for the metabolism of skatole and androstenone and thereby for the occurrence of boar taint.

1.3.1 Skatole metabolising enzymes

Skatole is described as having a faecal-like odour and is a product of microbial breakdown of tryptophan in the intestine of the pig. Once produced, a part is excreted with the faeces, while another part is absorbed through the gut wall, entering the bloodstream of the pig (Figure 2). Here it is transported via the portal vein to the liver, where it can be metabolised. If the hepatic clearance of skatole is insufficient, skatole accumulates in the adipose tissue.

Members of the CYP1A, 2A, 2C, 2E and 3A families have been studied in order to establish their importance for hepatic skatole clearance (Babol et al. 1998a; Diaz and Squires 2000a; Matal et al. 2009; Terner et al. 2006; Wiercinska et al. 2012). Taken together, these studies show that CYP2A19 and 2E1 are the most important ones, but that CYP1A2, 2C33 and 3A also metabolise skatole *in vitro*. It should also be noticed that aldehyde oxidase has been shown to be of importance for skatole clearance (Diaz and Squires 2000b). The importance of high CYP2A19 and 2E1 activity for the occurrence of boar taint is further supported by studies showing negative correlation with skatole concentrations (Squires and Lundström 1997; Zamaratskaia et al. 2006; Zamaratskaia et al. 2005; Zamaratskaia et al. 2012a). *In vitro* studies using primary porcine hepatocytes have shown that skatole induces CYP2A19 and 2E1 expression (Chen et al. 2008; Doran et al. 2002). This argues that knowledge about the regulation of the expression and activity of these CYPs is very important when working on finding a solution to the boar taint problem.

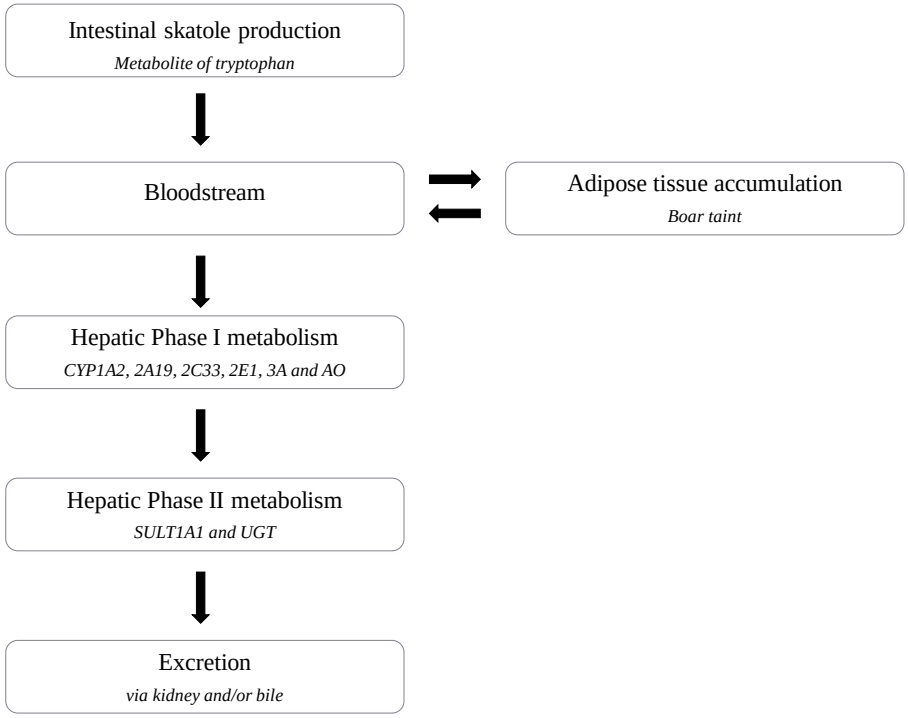

Figure 2. Skatole metabolism. Skatole is produced in the gastro-intestinal tract by microbial breakdown of the amino acid tryptophan and enters the bloodstream after absorption trough the intestinal wall. The hepatic clearance of skatole is conducted in two steps, ultimately resulting in excretion. If the hepatic clearance is insufficient, skatole accumulates in the adipose tissue. CYP, cytochrome P450; AO, aldehyde oxidase; SULT, sulfotransferase; UGT, uridine diphosphate glucuronosyltranferase.

There is strong support for a regulatory effect of steroids originating from the testis. CYP1A, 2A and 2E expression and/or activity has been shown to be low in entire (uncastrated) male pigs compared to females (Kojima et al. 2008; Skaanild and Friis 1999; Zamaratskaia et al. 2006) and surgically castrated pigs (Gillberg et al. 2006; Kojima et al. 2008; Niemelä et al. 1999; Skaanild and Friis 1999; Whittington et al. 2004; Zamaratskaia et al. 2009). Moreover, artificial manipulation off the hormone levels by immunocastration (immunization against gonadotropin-releasing hormone) (Brunius et al. 2012; Zamaratskaia et al. 2009) and injection with human chorionic gonadotropin (Zamaratskaia et al. 2008; Zamaratskaia et al. 2012b; Zamaratskaia et al. 2010) or steroids (Kojima et al. 2008; Kojima et al. 2010) has been shown to modify CYP expression and/or activity. Additionally *in vitro* studies, treating primary porcine hepatocytes with steroids, have likewise shown decreased CYP expression in the presence of androstenone (Chen et al. 2008; Doran et al. 2002). It should also be noticed that the presence of steroids has been shown to decrease the CYP

dependent activity *in vitro* (Rasmussen et al. 2011; Zamaratskaia et al. 2007; Zamaratskaia et al. 2009; Zamaratskaia et al. 2011).

Seven phase I metabolites of skatole have been identified; the major ones being 3-OH-3-methylindolenine, 3-methyloxindole and 3-OH-3-methyloxindole (Babol et al. 1998a; Diaz et al. 1999), several of them being substrates for the conjugation with sulphate (conducted by sulfotransferase 1A1; SULT1A1) or glucuronic acid (Babol et al. 1998b; Diaz and Squires 2003).

The importance of Phase II in the clearance of skatole is not fully elucidated, given the fact that Phase I metabolism is often the rate limiting step (Parkinson 2001).

1.3.2 Androstenone metabolising enzymes

Androstenone is produced in the Leydig cells of the testis and is often described as having a urine-like odour. From the testis it enters the bloodstream, where it either *1)* enters the salivary gland; acting as a pheromone, *2)* accumulates in adipose tissue or *3)* enters the liver; where it is metabolised (Figure 3).

Androstenone production is controlled by the hypothalamic-pituitary-gonadal axis and increases with sexual maturity. Hepatic metabolism of androstenone is, like skatole metabolism, conducted in two phases. The phase I metabolising enzymes 3α-hydroxysteroid dehydrogenase (HSD) and 3β-HSD converts androstenone to 3α- and 3β-androstenol, respectively. It has been shown that the major part of androstenone is converted to 3β-androstenol (Doran et al. 2004; Sinclair et al. 2005). This is further supported by the finding that low hepatic 3β-HSD expression and activity is accompanied by high concentrations of androstenone i adipose tissue (Chen et al. 2007b; Moe et al. 2007; Nicolau-Solano et al. 2007). Contradictory, Nicolau-Solano and Doran (2008) found that treating primary porcine hepatocytes isolated from pigs (weighing > 92 kg) with androstenone, increased the 3β-HSD expression. The molecular regulation of the transcription of the porcine 3β-HSD gene has recently started to be elucidated (Dong et al. 2012), but is basically unknown. However, the transcription of the human counterpart is complex and involves many transcription factors like steroidogenic factor-1, STAT5 (STAT; signal transducers and activators of transcription), liver X receptor α and farnesoid X receptor (FXR) (Feltus et al. 1999; Simard et al. 2005; Stayrook et al. 2008; Xing et al. 2009).

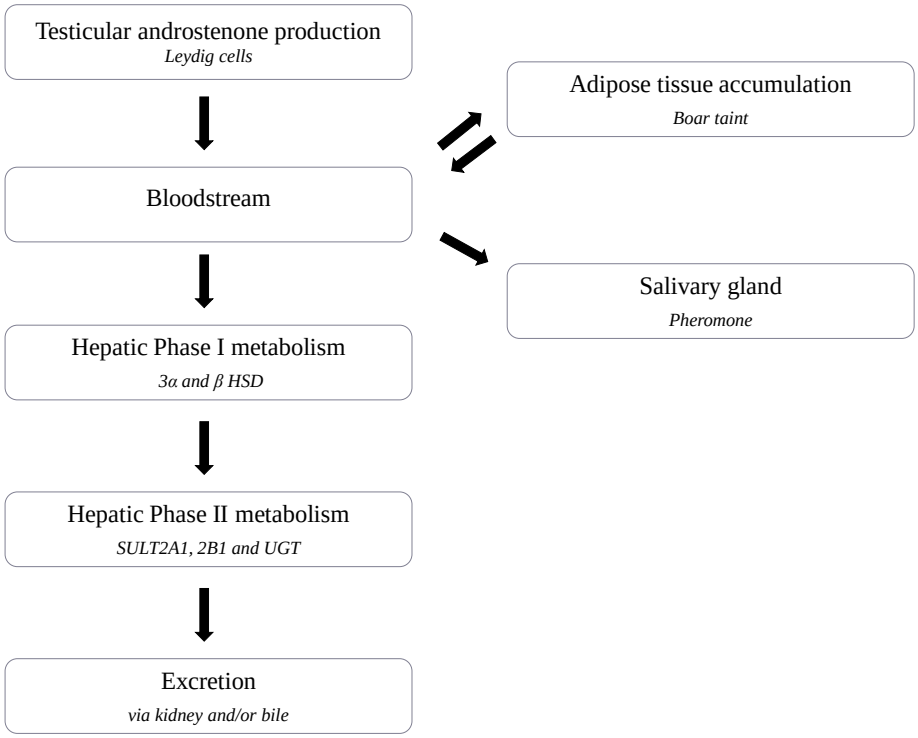

Figure 3. Androstenone metabolism. Androstenone is produced in the Leydig cells of the testis from where it enters the bloodstream. Via the bloodstream androstenone can enter the salivary gland; where it can be released as a pheromone. Androstenone is metabolised in the liver in two steps; if the hepatic clearance is insufficient it accumulates in the adipose tissue. HSD, hydroxysteroid dehydrogenase; SULT, sulfotransferase, UGT, uridine diphosphate glucuronosyltransferase.

In Phase II metabolism the androstenols are further converted to sulfoconjugated and glucuronidated metabolites (Sinclair et al. 2005). SULT2A1 and 2B1 together with UGT enzymes have been suggested to be involved in Phase II metabolism, however the involvement of SULT2B1 was shown to be breed dependent (Moe et al. 2007; Sinclair et al. 2006). Hepatic expression and activity of SULT2A1 has been shown to be higher in pigs with low androstenone concentrations (Sinclair et al. 2006). Knowledge about the molecular regulation of porcine SULT2A1 transcription is limited, though one study identified ligands of CAR, PXR and PXR/FXR as inducers of SULT2A1 activity *in vitro* (Sinclair et al. 2006).

1.3.3 Solutions to the boar taint problem

When raising sexually mature male pigs, > 10 % of them will carry boar tainted meat (meat with above 1.0 µg androstenone/mg fat and/or 0.25 µg skatole/mg fat) (Bonneau et al. 1992). In Denmark, boar tainted meat is paid a lower price per kilo at the slaughterhouse and is less accepted by the consumers. To avoid the occurrence of tainted meat, the common practice used by farmers in most European countries is surgical castration without anaesthesia of male pigs before the age of 7 days (Fredriksen et al. 2009). Surgical castration is effective in avoiding tainted meat, probably due to the removal of androstenone. Apart from the direct effect of androstenone on the sensory perception of the meat, it also contributes indirectly by its impact on CYP expression (Chen et al. 2008; Doran et al. 2002). The protein expression of CYPs is lowered by the presence of testicular steroids, especially androstenone, ultimately lowering the Phase I metabolism of skatole (Figure 4). Moreover a direct effect of androstenone on CYP activities has also been suggested (Rasmussen et al. 2011; Zamaratskaia et al. 2007; Zamaratskaia et al. 2009; Zamaratskaia et al. 2011).

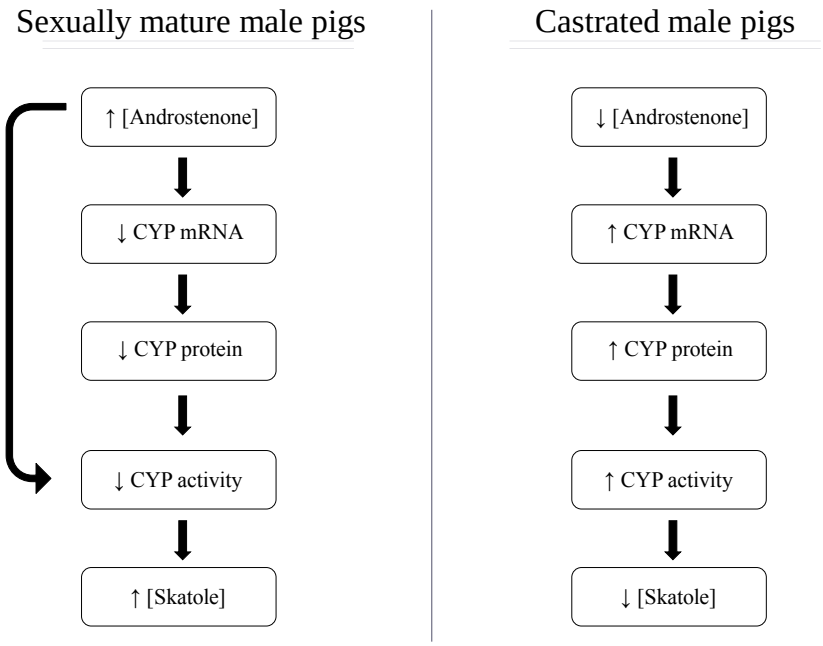

Figure 4. The impact of androstenone on skatole concentrations in entire and castrated male pigs. Increased androstenone concentrations in sexual mature male pigs lower the CYPs and thereby the CYP activity, giving rise to large skatole concentrations. Additionally, androstenone has a direct inhibitory effect on CYP activity. In castrated pigs, the production site of androstenone is removed; thereby abolish CYP inhibition, resulting in low skatole concentrations.

Due to negative animal welfare, castration of piglets has in recent years gained much focus by the public and politicians. Surgical castration of piglets without anaesthesia is associated with pain, discomfort and stress (von Borell et al. 2009). Motivated by animal welfare, Norway and Switzerland have started to take action, banning all surgical castration from 2009 and 2015, respectively. A similar ban is on its way in Denmark as well. Within EU there are also initiatives to end surgical castration. Several actors of the pig industry have signed a declaration committing them to work on ending all castration in 2018 and as a first step to use analgesia and/or anaesthesia from 2012. With that in mind, an alternative to the common practice of surgical castration without anaesthesia is highly needed.

A detailed and complete description of all alternatives to surgical castration without anaesthesia is not within the scope of this thesis, but can be found in von Borell *et al* (2009). Of the alternatives to castration, production of entire males, castration with anaesthetics and/or analgesia, immunocastration, genetic selection and feeding strategies are the most likely to be used in the future in order to reduce the occurrence of boar taint.

From a production point of view, it is more beneficial to produce entire male pigs compared to castrates, due to better growth rates, consumption of less feed and leaner carcasses (Lundström et al. 2009). However, raising entire males is not without concerns for animal welfare, due to increased aggressive and sexual behaviour causing problems in the production systems used today. Still, raising entire males can be an alternative to surgical castration, possibly in combination with *1)* slaughter at a lower weight (less sexual maturity), *2)* detection of tainted meat at the slaughterhouse and *3)* improved processing of the tainted meat. The use of anaesthetics and analgesia in combination with surgical castration has been shown to effectively reduce pain during and after castration (Hansson et al. 2011). But the administration of the anaesthesia and analgesia causes extra handling of the piglet, which might increase stress. Concerns about the effectiveness and safety of the anaesthetics have been raised (von Borell et al. 2009). Likewise, the use of drugs is incompatible with production of organic pig meat. Immunocastration is used in Australia and New Zealand to prevent boar taint. By immunising the pigs against Gonadotropin-releasing hormone the hypothalamic-pituitary-gonad axis is disturbed, causing low levels of steroid production in the testis. The effect of immunocastration on Phase I and II metabolising enzymes (Brunius et al. 2012; Rasmussen et al. 2012; Tomankova et al. 2012; Zamaratskaia et al. 2009), as well as boar taint compounds (Brunius et al. 2011) has shown that it can be an effective alternative. The major drawbacks off immunocastration are the safety concerns for the people administrating

the vaccine and the attitudes of the consumers towards meat from immunized pigs. Another approach to solving the boar taint problem is genetic selection and identification of relationships between the genetic profile and boar taint compounds (Gregersen et al. 2012; Grindflek et al. 2011a; Grindflek et al. 2011b; Moe et al. 2009). At present, work is still on-going, but the results are inconsistent between races.

1.3.4 Feeding strategies to prevent boar taint

One of the most easily implementable strategies to prevent boar tainted meat is the use of specially designed feeding. Experimental diets enriched with lupine (Aluwe et al. 2009; Hansen et al. 2008), Brussels sprouts (Hansen et al. 2002) and fishmeal (Lanthier et al. 2006) have all been investigated for their impact on the occurrence of boar taint, either by sensory scores or chemical analysis of skatole and androstenone. Likewise different additives have also been tested, including clinoptilolite (Aluwe et al. 2009), garlic (Leong et al. 2011), essential oils (Janz et al. 2007), enzyme supplementation (Pauly et al. 2011) and active carbon (Jen and Squires 2011). Taken together these studies show that the levels of skatole and androstenone in pigs can be manipulated by dietary ingredients or additives.

In the attempt to use special designed diets to lower the occurrence of boar taint, the most studied group of components is polysaccharides, like inulin (e.g. from chicory) and starch (e.g. from potatoes). The impact of including polysaccharides in the feed on skatole and androstenone levels in entire male pigs are summarized in Table 1.

The original motivation for investigating the effect of polysaccharides on skatole accumulation in pigs is due to its effect on the microbial production of skatole and/or intestinal skatole absorption. In the case of chicory or inulin it is suggested that the addition of polysaccharides would *1)* have pre-biotic affect; changing the microbial flora to one with lower potential for skatole production, *2)* decreased transition time in the gut; lowering time for skatole absorption; *3)* lowering of available tryptophan in the diet, thus the substrate for skatole production. Taken together the studies referred to in Table 1 shows that skatole levels are lower in pigs fed diets enriched in polysaccharides, while the effect on androstenone is more ambiguous.

Table 1. Effect of polysaccharides-based feed on skatole and androstenone in plasma and fat from entire male pigs

Polysaccharide source	Effect on skatole	Effect on androstenone	Notes	Reference
Chicory root	↓ fat	ND		(Claus et al. 1994)
	↓ fat	↓ fat	No effect of pure inulin on androstenone	(Byrne et al. 2008)[a]
	→ fat → plasma	ND		(Hansen et al. 2008)
	↓ fat	ND	< 6 % inulin no effect on skatole	(Kjos et al. 2010)*[b]
	↓ fat	↑ fat		(Zammerini et al. 2012)*
Pure inulin	↓ fat	ND	Prepubescent pigs	(Lanthier et al. 2006)
	→ fat	→ fat	No effect of raw potato starch	(Aluwe et al. 2009)
Jerusalem artichoke	↓ fat †	↓ fat †		(Vhile et al. 2012)
Raw potato starch	↓ fat	ND	→ CYP2A and 2E1 activity	(Zamaratskaia et al. 2005)
	↓ fat	ND	→ CYP2A and 2E1 activity	(Zamaratskaia et al. 2006)
	↓ fat → plasma	→ fat	↓ unconjugated plasma androstenone	(Chen et al. 2007a)
	↓ fat → plasma	→ fat		(Pauly et al. 2008)[c]
	↓ fat	↓ fat †		(Øverland et al. 2011)
Sugar beet pulp	→ fat	ND	No effect of wheat bran or soybean	(Van Oeckel et al. 1998)
	↓ plasma	ND		(Knarreborg et al. 2002)
	↓ fat	→ fat	↑ CYP2E1 protein expression	(Whittington et al. 2004)

Significant ↑ increased, ↓ decreased or → unchanged concentrations compared to control. * Studies using processed chicory root products. The same results are published in; [a] Hansen *et al* (2006); [b] Øverland *et al* (2011) and [c] Pauly *et al* (2010). ND, not determined. † tendency ($p < 0.1$) towards significant changes.

Several studies have shown that CYP expression and activity is changed with the administration of plant materials and herbal based medicine (Chang 2009; Chang and Waxman 2006; Delgoda and Westlake 2004). These effects are often shown to be caused by the presence of XR ligands, e.g. coumarines and sesquiterpenes. Thus, in addition to the above mentioned effects of chicory on skatole accumulation in pigs, it has been suggested that bioactive compounds in chicory, like sesquiterpene lactones, are of importance (Hansen et al. 2006). However, a link between

sesquiterpene lactones and skatole accumulation was not further explained by the authors. Sesquiterpene lactones are a major class of plant secondary metabolites, possessing biological activities like anti-tumour effect, anti-bacterial activity as well as pharmacological activity (Bais and Ravishankar 2001; Picman 1986; Rodriguez et al. 1976). In chicory a number of different sesquiterpene lactones have been described; the major ones being lactucin, 8-deoxylactucin and lactucopicrin (Cavin et al. 2005; de Kraker et al. 2002; Kisiel and Zielinska 2001). The sesquiterpene lactone artemisinin (from Artemisia) has been shown to induce CYP expression in mice, rats and human hepatocytes, probably by activation of CAR and/or PXR (Burk et al. 2005; Simonsson et al. 2006). Moreover, chicory contains coumarines, like esculetin and esculin (Bais and Ravishankar 2001). Scoparone (another coumarin) has also been shown to increase the expression of CYP in mice and human hepatocytes, probably by activating CAR (Huang et al. 2004; Yang et al. 2011). This suggests that chicory might affect the expression and activity of hepatic Phase I and II enzymes.

1.4 Methods for studying liver metabolism

The study of hepatic Phase I and II metabolism is essential for the pharmaceutical industry and of most importance when developing new drugs (Brandon et al. 2003). This has impelled the development of *in vitro* models to resemble the *in vivo* metabolism (Guillouzo 1998). Ultimately, the most optimal system would be to use whole animals, feed them with the experimental diet and afterwards study changes in hepatic metabolism. However, this is not always possible due to costs, time and ethical aspects. Additionally, whole animals are complex in their response, so less complex model systems can be useful in the study of more isolated events and for screening studies. Moving from the whole animal towards less complex model systems could be as follows: perfused whole livers, liver slices, primary hepatocytes, cell lines, microsomes and finally recombinant enzymes (Brandon et al. 2003). It is beyond the scope of this thesis to describe all model systems and their pros and cons, however, due to its use in the experimental work some comments about microsomes and cell cultures are included.

Microsomes consist of vesicles of the endoplasmic reticulum made by mechanical disruption of the cells, followed by isolation using centrifugation. As a result, they lack cytosolic enzymes, needs cofactors for activity, but are rich in CYPs and UGTs. They are extensively used in the pharmaceutical industry for studying biotransformation and substrate metabolites (Brandon et

al. 2003). Likewise microsomes are often used as sample preparation when determining changes in CYP expression and/or activity

Cell models are likewise often used to study hepatic metabolism. Cell models can be divided into cell lines (immortalized cells often isolated from tumours) or primary cells (isolated from normal tissue). They are rather complex models suitable for studies of cellular adaptations to a given treatment. A number of commercially available cell lines, including HepG2, have been used to study liver metabolism, however they are often not representative to the normal cell, with regard to constitutive protein expression and XR function. In contrast, primary hepatocytes resemble the *in vivo* metabolism to a high degree, although they lose some function with time in culture. In general the use of cell models is essential in the study of dynamic changes with treatment, including mRNA and protein induction.

2.0 Objective

Previous studies have investigated the effect of including chicory root in the feed to pigs. These studies investigated, among other things, the impact of chicory on bacterial population in the gastrointestinal tract, on skatole accumulation and the sensory perceptions of the meat. Taken together these studies put forward the hypothesis that dietary chicory decreases skatole accumulation in fat and thereby improve the sensory perception of the meat. The mechanism behind this is not fully elucidated; one possible explanation might be that bioactive compounds present in chicory increases the hepatic skatole clearance. Additionally, studies have shown unclear results on the effect of chicory on androstenone accumulation. Thus the objective of this PhD study were to further elucidate the impact of chicory on boar taint, by investigating the effects on hepatic Phase I and II enzymes relevant for skatole and androstenone metabolism. As boar taint usually is associated with meat from sexually mature male pigs, a comparison of constitutive expression of hepatic Phase I enzymes between female and entire male pigs can add new knowledge on the importance of liver metabolism on boar taint.

2.1 Hypothesis

It is hypothesized that bioactive compounds in chicory act as inducers of hepatic Phase I and II enzyme of importance for skatole and androstenone metabolism. By increasing the hepatic skatole and androstenone clearance, accumulation of these compounds will decrease.

3.0 Materials and methods

The following chapter serves as a summary of the experimental setups and methods used; more detailed descriptions are presented in the papers included in the thesis.

3.1 Animals, feeding and sampling

3.1.1 *In vivo* experiment

22 entire male pigs and 4 female pigs (Landrace x Yorkshire sire and Duroc boars) were included in the study (Figure 5). Of the 22 male pigs, 16 were randomly allocated to be fed dried chicory root (DCR; Table 2) for the last 16 days before slaughter. The last 6 served as control, being fed a standard diet for the last 16 days before slaughter (CON; Table 2). The 4 female pigs also received standard diet (CON; Table 2).

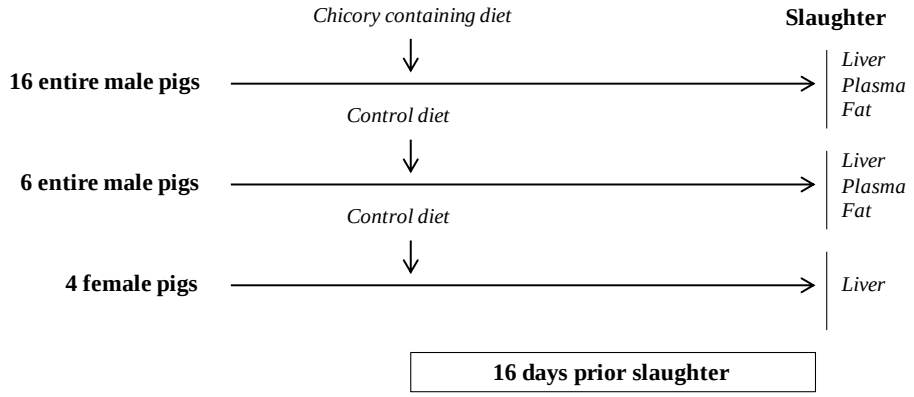

Figure 5. Time schedule for feeding and sampling in the *in vivo* experiment.

Both diets were energy matched and offered *ad libitum*. All pigs were slaughtered at the same day, at an age of 164 days. Details about slaughter and sampling are given in Paper III. During sampling at the slaughterhouse, liver samples from two chicory fed pigs were lost. Sample materials from the pigs were used in Paper I-V.

Table 2. Composition and nutrient content of the diets used in the feeding experiment

	CON	DCR
Components (% of total weight)		
Dried chicory root[a]	-	10.0
Barley	60.0	50.0
Soybean cake	16.7	19.0
Wheat	12.1	11.3
Oat	5.0	3.0
Vegetable fat	1.9	1.2
Melasses	1.0	3.2
Calculated analysis (g/kg)		
FEsvin[b]	1.0	1.0
Dry matter	863.6	871.3
Crude protein	156.0	161.8
Crude fat	42.2	32.0
Total fibre	165.1	226.1
Inulin	-	47.1
Tryptophan	2.0	2.1

CON, control diet; DCR, dried chicory root diet. [a] Dried at 65 °C. [b] Feeding unit pig. From Paper III.

3.1.2 *In vitro* experiment

Livers from five female piglets (Landrace x Yorkshire sire and Duroc boars; 11.1 ± 0.5 kg) were used for preparation of primary porcine hepatocytes as described in Paper VI. The piglets were slaughtered at Aarhus University (Foulum), ensuring quick and easy transfer to lab-facilities for isolation of hepatocytes. In preliminary experiments the protocol described in Paper VI was established; optimizing isolation buffers, media and extra-cellular matrix. Moreover, incubation time for inducers/treatments and conditions for the control samples were optimized. The time schedule used to study regulation of mRNA expression is shown in Figure 6.

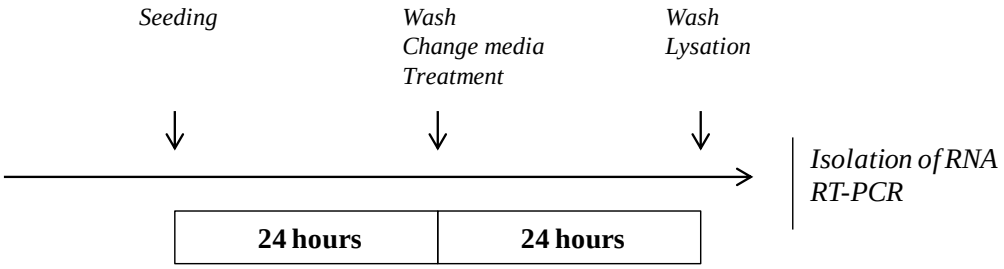

Figure 6. Time schedule studying regulation of CYP mRNA expression *in vitro* using primary porcine hepatocytes. RT-PCR, real time polymerase chain reaction.

The hepatocytes were treated with various standard CYP inducers, secondary plant metabolites and chicory root, according to table 3. The chosen standard CYP inducers were selected for their ability to induce AhR, CAR and PXR regulated CYPs.

Table 3. Compounds used for *in vitro* treatment of primary hepatocytes and their expected action

	Compound	Concentration	Solvent	Action
Standard CYP inducers				
	β-naphthoflavone	100 µM	DMSO	AhR ligand
	CITCO	100 µM	DMSO	Human CAR ligand
	TCPOBOP	5 nM	DMSO	Murine CAR ligand
	Dexamethasone	50 µM	DMSO	PXR activation
Secondary plant metabolites				
	Artemisinin	1-100 µM	DMSO	CAR/PXR activation
	Scoparone	10-100 µM	DMSO	CAR activation
Extract				
	Chicory root	1-100.000 arbitrary units	Methanol	Unknown

CITCO,6-(4chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbalehyde O-(3,4-dichlorobenzyl)oxime; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene; DMSO, dimethyl sulfoxide; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; PXR, pregane X receptor.

3.2 Preparation of hepatic microsomes

For preparation of hepatic microsomes, liver samples were minced and subsequently disrupted on a motor driven homogenizer (Polytron 2100). Following an initial 10.000 *g* centrifugation for 10 min, two different methods were used for isolation of the microsomes; ultracentrifugation (100.000 *g*) or Ca^{2+} aggregation/low-speed centrifugation (25.000 *g*). In addition two different buffers were used; Tris-sucrose or Tris-EDTA. Details about methods and buffers are given in Paper I. Microsomes used in Paper II-V were prepared by ultracentrifugation.

Protein concentrations were determined using the Pierce® BCA protein kit (Thermo scientific) with bovine serum albumin as standard.

3.3 mRNA expression

Total RNA from the samples was isolated using a commercial available RNA isolation kit (Rnasey Mini Kit, WVR, Denmark). For RNA isolation of liver samples, frozen tissue was first homogenized in lysis-buffer (provided with the kit), while for RNA isolation from primary hepatocytes, the cells were lysed in the well using lysis-buffer.

The specific mRNA expression was determined using reverse transcriptase and real time polymerase chain reaction (PCR) with TaqMan probes, as described in Paper II. All analysis were conducted in accordance with the guidelines given by Huggett *et al* (2005), suggesting *1)* isolation of RNA from similar amounts of tissue, *2)* converting similar amounts of RNA into cDNA and *3)* normalizing to a reference gene. For calculation of the relative mRNA expression, the efficiency of the PCR was always determined, by running a serial dilution of a cDNA sample. The relative mRNA expression was calculated using Equation 1 (Pfaffl 2001);

Equation 1:

$$ratio = \frac{(E_{target})^{\Delta Ct_{target}(control-sample)}}{(E_{housekeeping})^{\Delta Ct_{housekeeping}(control-sample)}}$$

Where:

E_{target}	=	*real time PCR efficiency of the target gene*
$E_{housekeeping}$	=	*real time PCR efficiency of the reference gene*
Ct	=	*numbers of PCR cycles to reach threshold*
ΔCt_{target}	=	*Ct-value of the target gene in the control sample – Ct-value of the target gene in the treated sample*
$\Delta Ct_{housekeeping}$	=	*Ct-value of the target gene in the control sample – Ct-value of the target gene in the treated sample*

The chosen housekeeping genes were glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and β-actin. The obtained Ct-values for both GAPDH and β-actin were not affected by any of the treatments or gender. As the Ct-values for GAPDH showed least variation, it was chosen as the standard housekeeping gene. However, initial data analysis using β-actin showed the same trend in the results as GAPDH. Primer and probe sequences for GAPDH and β-actin are given in Young *et al* (2007) and Theil *et al* (2006), respectively.

Primers and TaqMan probes were designed with Primer Express (Version 2, Applied Biosystems) using pig specific sequences of genomic DNA (http://www.ensembl.org/Sus_scrofa/Info/Index?). To verify that the primers and probes were specific, a nucleotide search using NCBI's Basic Local Alignment Tool (http://blast.ncbi.nlm.nih.gov/) was performed. Primers and probes were subjected to samples of genomic DNA as well as H_2O, showing no amplification with PCR. Finally, the product from a PCR run was separated on an agarose gel, confirming that only one amplicon was present, having the desired base pair length, and that no primer-dimer formation existed. The used primers and probes are given in Table 4.

3.4 Protein expression

The relative protein expression was determined by Western blotting as described in Paper I. All samples to be compared were loaded on the same gel with normalised total protein concentrations. To quantify the occurring bands on the blots, both their size and intensity was taken into account.

Primary antibodies are given in Table 4, while secondary antibodies with a conjugated Alexa Flour 488 (DAKO, Denmark) were specific towards the species of the animal producing the primary antibody. For analysis of CYPs and 3β-HSD expression, microsomes were used, while SULT2A1 was analysed in a total protein precipitate from the cytosolic phase originating from the preparation of the microsomes.

3.5 Cytochrome P450 dependent activities

The CYP dependent activity was measured in microsomes as the rate of production of substrate specific metabolites. The substrates used are given in Table 4. Details on the incubation and determination of substrate products are given in Paper II, III and V.

To quantify the amount of substrate specific metabolites produced, the results were related to a standard curve. The CYP activity is presented as absolute values, given as the production of specific substrate metabolites in pmol/min/mg total protein.

Table 4. Summary of primers and probes for PCR, antibodies for Western Blotting and substrates for activity measurements and their use

	PCR	**Western blotting**	**Activity**	**Use**
	Primers and TaqMan probes (5´-3´)	Antibody	Substrate	Paper
CYP				
1A2	*F:* CTGCAATTCCTGAGGAAAATGG *R:* CGCTTGTGATGTCCTGGATACA *T:* AGGAGCGCTATCGGGACTTTGACAAGAA	SP1320A (Acris Antibodies)	Ethoxyresorufin Methoxyresurofin	I, II, III, VI
2A19	*F:* TGGATGAGAACGGGCAGTTT *R:* AGAGCTCCATTCTAGCCAGACCTT *T:* TCTCCATCGGAAAGCGGTACTGTTTCG	SC-53615 (Santa Cruz)	Coumarin	I, II, III, VI
2C33	*F:* TGGGAATCTGATGCAACTTAACC *R:* AACAGGGCCGTACTGTTTGG *T:* AAGGACATCCCTGCGTCTCTTTCCAAGTT	ND	Tolbutamide	V, VI
2D25	*F:* TTGGAAGGACTGAAGGAGGAAGT *R:* CCCGGGATATGCCTGAGAA *T:* CCTCATGCGCCAGGTGCTGGA	ND	ND	VI
2E1	*F:* CGGAAAGTTCAAGTACAGTGATCATT *R:* GGCCCTCTCCGACACACA *T:* CAAGGCATTTTCCGCAGGAAAGCG	Ab28146 (Abcam)	*p*-Nitrophenol	I, II, III, VI
3A29[a]	*F:* GGACACCATAAATCCTTACACTTACCT *R:* GCAAACCTCATGCCAATGC *T:* CCTTTGGGACTGGACCCCGCAA	ND	Benzyloxyresorufin Benzyloxyquinoline	V, VI
Other				
AhR[b]	*F:* AGCTGCACTGGGCGTTAAA *R:* GCCACTCGCTTCATCAATTCT *T:* CCTTCACAGTGTCCAGACTCTGGAC	ND	ND	V
CAR[a]	*F:* TTCATCCATCACCAGCACTTG *R:* TGATGTCCGCGAAATGCA *T:* CCCTGGTGCCTGAACTGTCTCTGCTC	ND	ND	V
PXR[a]	*F:* GCTGAACTGTGCTAGGCTTCTG *R:* CCTCCCACGAGCCATGTT *T:* ATGCACCGGGACACAAGTGAGGG	ND	ND	V
3β-HSD	*F:* GGGCGAGAGACCGTCATG *R:* ACGCTGGCCTGGACACA *T:* AGGTCAATGTGAAAGGTACCCAGCTC	R1484 (J.I. Mason, Uni. Edinburgh)	ND	IV
SULT2A1	*F:* TTCCAGGAGAAGATGGCAGATC *R:* AGGATTGGGAAGTTTGTGAACATT *T:* TCCTCAAGAGCTGTTCCCATGGCAA	SC-46528 (Santa Cruz)	ND	IV

F, forward primer; R, reverse primer; T, TaqMan probe; ND, not determined. [a] primers and probes designed by Nannelli *et al* (2010); [b] primers and probes designed by Messina *et al* (2009). CYP, cytochrome P450; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; PXR, pregnane X receptor; HSD, hydroxysteroid dehydrogenase; SULT, sulfotransferase.

3.6 Analysis of boar taint compounds and hormones

Plasma concentrations of testosterone and estradiol were analysed using commercially available kits; RIA (TKTT, Diagnostic Products Corporation, Webster, TX, USA) and EIA (1-3702, Salimetrics, State College, PA, USA), respectively, according to Paper IV. Likewise insulin-like growth factor 1 (IGF-1) concentrations were analysed using a commercially available kit (EIA, DSL-10-2800, Diagnostic Products Corporation, Webster, TX, USA), according to Paper IV. Determination of skatole and indole in plasma and adipose tissue was performed using HPLC, according to Paper IV. Likewise androstenone concentrations in adipose tissue were analysed using HPLC, according to Paper IV.

The absolute concentration of the analysed compounds was obtained by relating the results to obtained standard curves.

3.7 Statistics

In Paper I a GLM procedure was used to evaluate differences. For pair-wise comparisons in Paper II-V, Student's unpaired t-test was used. For comparison of multiple groups in Paper II, ANOVA, with Tukey's post-hoc test to identify differences, was used. In Paper VI, two-way ANOVA was used to evaluate the effect of time in culture in freshly and cryopreserved hepatocytes, one-way ANOVA followed by Tukeys post-hoc test were used to evaluate the effect of standard XR activators and secondary plant metabolites. To evaluate the effect of the chicory extract on mRNA expression relative to control, Students t-test with Bonferroni's correction was used.

4.0 Results

4.1 Microsomes – comparison of preparation methods

The protein concentrations measured with Western blotting was highest in the microsomes prepared with ultracentrifugation, while there was no effect of the buffer used (Table 5). Due to large variation in the constitutive CYP protein expression between the used pigs, the results for CYP1A2 and 2E1 protein concentrations only almost reached statistical significance. All measured CYP dependent activities were highest in microsomes prepared by ultracentrifugation, apart from coumarin hydroxylation.

Table 5. Concentration and activity of cytochrome P450 in hepatic microsomes prepared by two different methods using two different buffers

Method	**Ultracentrifugation**		**Ca^{2+}-aggregation**		Std. error	*p*-value method	*p*-value buffer	*p*-value buffer x method
	TRIS-sucrose	*TRIS-EDTA*	*TRIS-sucrose*	*TRIS-EDTA*				
CYP1A2†	138.0	154.2	100.0	111.7	20.60	0.07	0.51	0.91
CYP2A6†	126.6	124.4	100.0	99.7	11.38	0.04	0.91	0.93
CYP2E1†	122.5	133.4	100.0	110.9	12.49	0.09	0.39	1.00
EROD	137.8	142.2	104.4	109.6	13.76	0.03	0.73	0.98
MROD	21.3	19.6	15.1	16.3	1.72	0.01	0.89	0.42
PROD	7.9	6.5	5.4	5.6	0.70	0.02	0.38	0.26
COH	87.7	78.4	62.1	60.9	21.78	0.33	0.81	0.85
PNPH	790.5	747.0	507.4	591.2	78.69	0.01	0.80	0.43

All data presented as least-squares means. EROD, 7-ethoxyresorufin O-deethylase; MROD, 7-methoxyresorufin O-demethylase; PROD, 7-pentoxyresorufin O-depentylase, COH, coumarin hydroxylase; PNPH, p-nitrophenol hydroxylase. All activities are given as pmol/min/mg total protein. † arbitrary protein concentration measured using Western blotting. From Paper I.

4.2 *In vivo* effects of chicory root

Inclusion of chicory in the feed had no effect on the apparent health of the pigs, as judged by the use of medicine during growth and a visual inspection at the slaughterhouse. There was no significant difference in carcass weight between the groups (chicory fed male pigs, 93.0 ± 9.3 kg; control fed male pigs, 94.2 ± 7.3 kg; female pigs, 91.3 ± 2.7 kg).

4.2.1 mRNA expression of hepatic XRs and Phase I and II enzymes

There was no effect of chicory feeding on the mRNA expression of the XRs: AhR, CAR and PXR. Of the investigated Phase I enzymes increased mRNA levels were found for CYP1A2, 2A19, 2D25, 2E1, 3A29 and 3β-HSD in chicory fed pigs (Figure 7). Feeding with chicory had no effect on the mRNA expression of CYP2C33 and SULT2A1.

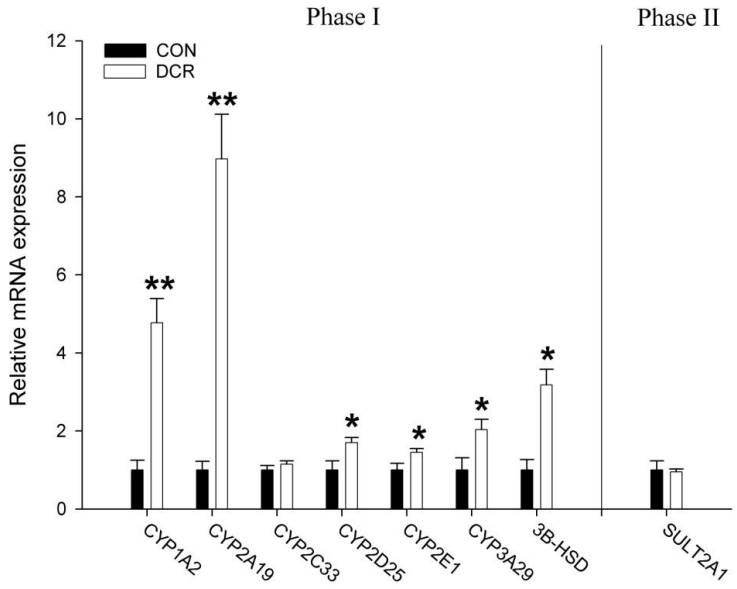

Figure 7. Hepatic cytochrome P450, 3β-HSD and SULT2A1 mRNA expression in control (CON) and chicory (DCR) fed entire male pigs. Data are presented as mean ± SEM. Levels of significance: ** $p < 0.01$; * $p < 0.05$. CYP, cytochrome P450; HSD, hydroxysteroid dehydrogenase; SULT, sulfotransferase. From Paper III, IV and V. Data on CYP2D25 expression are not published.

4.2.2 Protein expression of hepatic Phase I and II enzymes

Protein expression CYPs and 3β-HSD was determined by Western blotting using microsomes, while SULT expression was determined in a precipitate of proteins from the cytosol. Of the investigated Phase I enzymes increased protein levels were found the chicory fed pigs for CYP1A2, 2A19 and 3β-HSD (Figure 8). There was no effect of chicory feeding on the expression of CYP2E1 and SULT2A1.

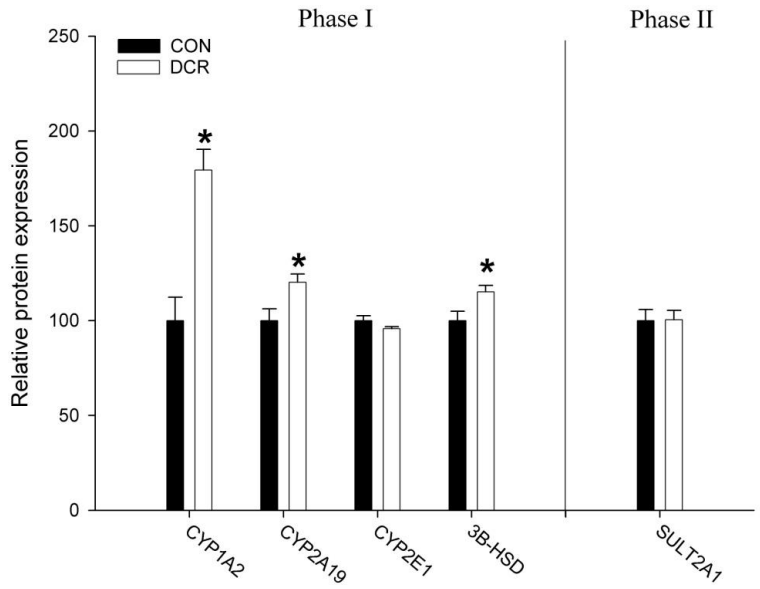

Figure 8. Hepatic cytochrome P450, 3β-HSD and SULT2A1 protein expression in control (CON) and chicory (DCR) fed entire male pigs. Data are presented as mean ± SEM. Levels of significance: ** $p < 0.01$; * $p < 0.05$. CYP, cytochrome P450; HSD, hydroxysteroid dehydrogenase; SULT, sulfotransferase. From Paper III and IV.

4.2.3 Activity of hepatic Phase I enzymes

The specific CYP dependent activities were determined in microsomes using specific probe substrates. Of the investigated activities, methoxyresorufin O-demethylase (CYP1A2), coumarin hydroxylation (CYP2A), 7-benzyloxyresorufin O-dealkylase (CYP3A) and 7-benzyloxyquinoline O-debenzylase (CYP3A) was increased in chicory fed pigs (Table 6). Ethoxyresorufin O-deethylase (CYP1A1), tolbutamide hydroxylation (CYP2C) and *p*-nitrophenol hydroxylation (CYP2E1) were not affected by chicory feeding.

Table 6. Hepatic cytochrome P450 dependent activity in control (CON) and chicory (DCR) fed entire male pigs

CYP subfamily	**CYP- dependent activity**			
	Reaction	CON	DCR	
1A	*EROD*	38.1 ± 4.6	39.1 ± 3.8	
	MROD	8.2 ± 0.6	12.2 ± 0.8	**
2A	*COH*	3.0 ± 1.1	13.0 ± 1.7	*
2C	*TBOH*	26.0 ± 4.2	104.7 ± 25.9	
2E	*PNPH*	20.6 ± 9.1	29.5 ± 3.9	
3A	*BROD*	6.6 ± 1.5	13.6 ± 2.0	*
	BQOD	297.1 ± 75.9	919.9 ± 147.0	*

Data are presented as mean pmol/min/mg total microsomal protein ± SEM. Levels of significance: ** $p < 0.01$; * $p < 0.05$. EROD, ethoxyresorufin O-deethylase; MROD, methoxyresurufin O-demethylase; COH, coumarin hydroxylation; TBOH, tolbutamide hydroxylase; PHPH, *p*-nitrophenol hydroxylation; BROD, 7-benzyloxyresurofin O-dealkylase; BQOD, 7-benzyloxyquinoline O-debenzylase. From Paper III and V.

4.2.4 Boar taint compounds and hormones

There was no difference between the concentrations of skatole found in plasma and fat of control and chicory fed pigs (Table 7). The concentration of indole was increased in fat from chicory feed pigs, while no difference was found in plasma between the two groups. The chicory fed group of pigs had lower concentrations of androstenone in fat, compared to the control fed pigs.

Table 7. Concentrations of skatole, indole and androstenone in fat and plasma from control (CON) and chicory (DCR) fed entire male pigs

Sample side	Compound	CON	DCR	
Fat	Skatole	49.3 ± 19.6	44.2 ± 4.1	
	Indole	24.7 ± 7.8	59.3 ± 6.7	*
	Androstenone	2.3 ± 0.7	0.9 ± 0.1	*
Plasma	Skatole	4.1 ± 1.0	5.5 ± 0.8	
	Indole	3.5 ± 0.5	5.3 ± 0.8	

Data are presented as mean ng/ml ± SEM. Levels of significance: ** $p < 0.01$; * $p < 0.05$. From Paper IV.

The plasma concentrations of testosterone, estradiol and IGF-1 were not different between the control and chicory fed pigs (Table 8).

Table 8. Concentrations of testosterone, estradiol and insulin-like growth factor 1 (IGF-1) in plasma from control (CON) and chicory (DCR) fed entire male pigs

Compound	CON	DCR
Testosterone (ng/ml)	7.8 ± 1.9	5.1 ± 1.0
Estradiol (pg/ml)	24.9 ± 10.7	10.3 ± 2.5
IGF-1 (ng/ml)	343.8 ± 63.9	394.4 ± 22.1

Data are presented as mean ± SEM. From Paper IV.

4.3 *In vitro* effect of chicory root on CYP mRNA expression

Isolation and culturing of primary hepatocytes were not established at Department of Food Science, Aarhus University, when the project started. For that reason, it was necessary to develop a protocol for isolation and culturing of primary hepatocytes, and to evaluate their performance in culture and response to cryopreservation before use.

The constitutive mRNA expression with time in culture was decreased for CYP1A2, 2A19, 2D25 and 2E1, while no changes was shown for 2C33 and 3A29. More importantly, no difference in the mRNA response between freshly isolated and cryopreserved hepatocytes was shown, suggesting that cryopreserved hepatocytes resemble freshly isolated with respect to gene regulation. It should be noticed that cryopreservation decreased the viability of the hepatocytes by approximately 20%.

4.3.1 Effect of standard CYP inducers

In order to establish the hepatocytes potential for CYP induction they were treated with standard activators of the XRs (Figure 9). β-naphthoflavone is commonly used to activate AhR, while CITCO and TCPOBOP are used to activate CAR. Dexamethasone is used to activate PXR. β-naphthoflavone increased the mRNA expression of CYP1A2. Likewise, CITCO increased the mRNA expression of CYP1A2. Dexamethasone increased the expression of CYP1A2 and 3A29, while the increase in 2C33 mRNA expression was almost significant ($p = 0.054$). TCPOBOP has no effect on CYP expression.

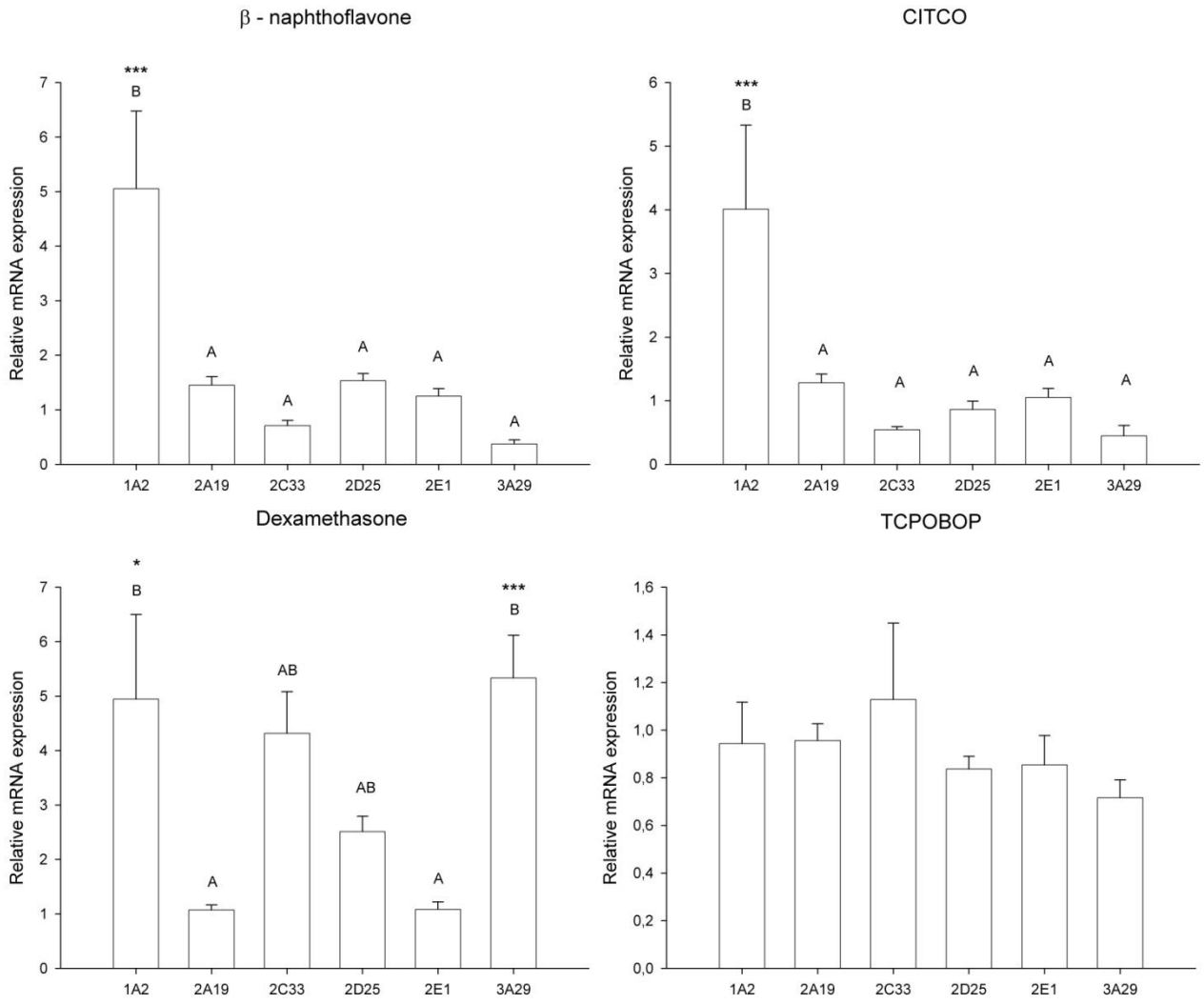

Figure 9. Cytochrome P450 isoform specific mRNA expression in hepatocytes treated with 100 µM β-naphthoflavone, 100 µM CITCO, 50 µM dexamethasone or 5 nM TCPOBOP for 24 hours. * $p < 0.05$; *** $p < 0.001$ significantly different from control samples (no inducer included). Bars not sharing subscription are significantly different from each other. Data are mean ± SEM relative to control samples. From Paper VI.

4.3.2 Effect of secondary plant metabolites

In order to determine the ability and the magnitude by which secondary plant metabolites like sesquiterpene lactones and coumarins affect CYP expression, the hepatocytes were treated with artemisinin and scoparone. Treating hepatocytes with artemisinin increased the mRNA expression of CYP1A2, 2C33, 2D25 and 3A29 (Figure 10), while treatment with scoparone increased the expression of CYP1A2 and 2D25 (Figure 10). The mRNA expression on CYP2A19 and 2E1 was not affected by artemisinin and scoparone.

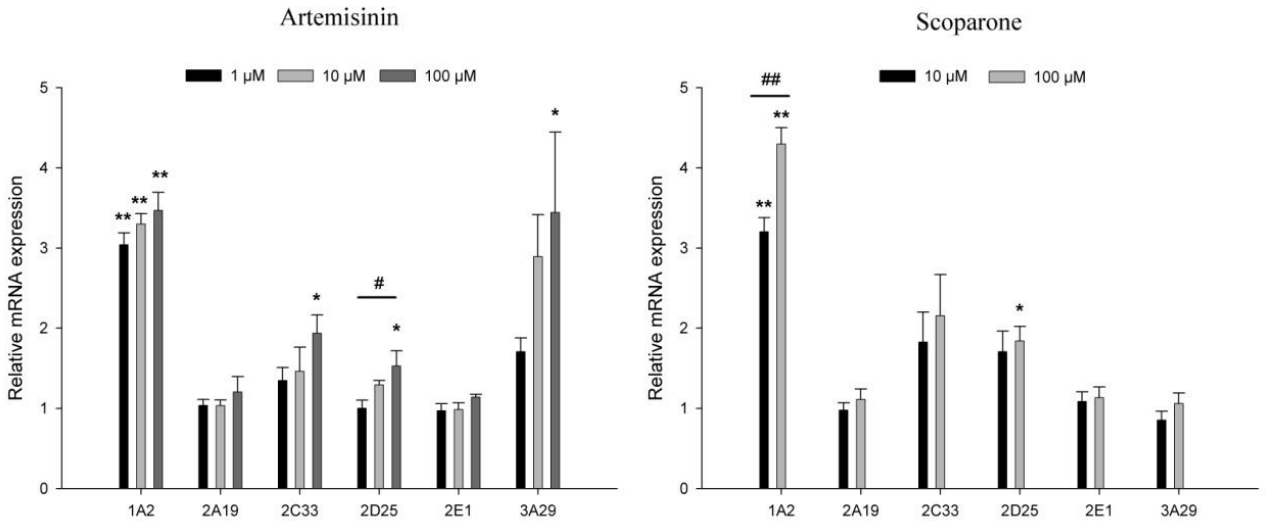

Figure 10. Cytochrome P450 isoform specific mRNA expression in hepatocytes treated with 1-100 µM artemisinin or 10-100 µM scoparone for 24 hours. * $p < 0.05$; ** $p < 0.01$ significantly different from control samples (no artemisinin or scoparone included). # $p < 0.05$; ## $p < 0.01$ significantly different from each other. Data are mean ± SEM relative to control samples. From Paper VI.

4.3.3 Effect of chicory root extract

The *in vitro* effect of chicory root was investigated by treating hepatocytes with different amounts of a methanolic extract of dried chicory root. Compared to control, high amounts of the chicory extract decreased the mRNA expression of CYP1A2, 2C33, 2D25 and 3A29 (Figure 11). The chicory extract had no effect on CYP2A19 and 2E1 mRNA expression.

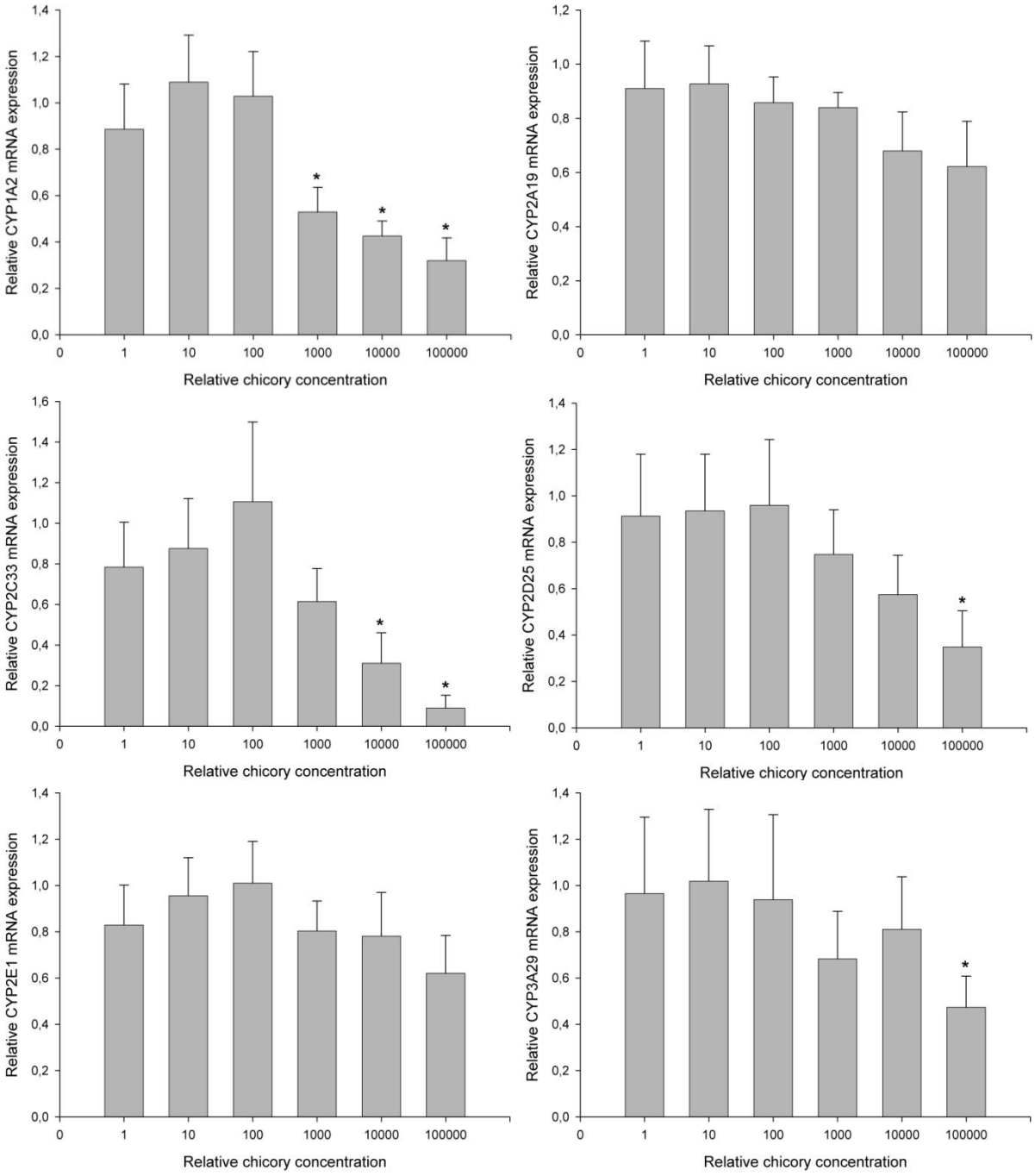

Figure 11. Cytochrome P450 isoform specific mRNA expression in hepatocytes treated with various amounts of chicory root extract for 24 hours. * $p < 0.05$ significantly different from control samples (no chicory extract included). Data are mean ± SEM relative to control samples. From Paper VI.

4.4 Gender-related differences in hepatic Phase I enzymes

Table 9. Gender-related differences in constitutive mRNA and protein expression of xenobiotic receptors and Phase I and II enzymes and cytochrome P450 dependent activities.

Omic-level	Gene/Enzyme	Male	Female	
mRNA expression (arbitrary units)	*AhR*	1.0 ± 0.2	1.5 ± 0.5	
	CAR	1.0 ± 0.2	1.6 ± 0.3	
	PXR	1.0 ± 0.2	1.2 ± 0.1	
	CYP1A2	1.0 ± 0.3	4.9 ± 0.6	**
	CYP2A19	1.0 ± 0.2	6.7 ± 2.2	*
	CYP2C33	1.0 ± 0.1	1.4 ± 0.3	
	CYP2D25 †	1.0 ± 0.2	1.8 ± 0.7	
	CYP2E1	1.0 ± 0.2	1.4 ± 0.3	
	CYP3A29	1.0 ± 0.3	2.1 ± 0.2	*
	3β-HSD †	1.0 ± 0.3	4.1 ± 0.4	**
	SULT2A1 †	1.0 ± 0.2	1.0 ± 0.1	
Protein expression (arbitrary units)	*CYP1A2*	100.0 ± 12.3	112.7 ± 4.7	
	CYP2A	100.0 ± 6.2	107.9 ± 5.7	
	CYP2E1	100.0 ± 2.6	93.3 ± 1.6	
Activity (pmol/min/mg total protein)	*CYP1A*	38.1 ± 4.6[a]	62.4 ± 6.0	**
		8.2 ± 0.6[b]	13.7 ± 1.7	**
	CYP2A	3.0 ± 1.1[c]	22.7 ± 7.4	*
	CYP2C	26.0 ± 4.2[d]	40.4 ± 2.3	*
	CYP2E1	20.6 ± 9.1[e]	41.0 ± 5.5	
	CYP3A	6.6 ± 1.5[f]	4.2 ± 0.5	
		297.1 ± 75.9 [g]	375.9 ± 71.6	

Data are presented as mean ± SEM. Measured as, [a] ethoxyresorufin O-deethylase; [b] methoxyresorufin O-demethylase; [c] coumarine hydroxylation; [d] tolbutamide hydroxylation; [e] *p*-nitrophenole hydroxylation; [f] 7-benzyloxyresorufin O-dealkylase; [g] 7- benzyloxyquinoline O-debenzylase. Levels of significance: * $p < 0.05$; ** $p < 0.01$. From Paper II and V. † not published.

Female pigs showed larger constitutive mRNA expression of CYP1A2, 2A19, 3A29 and 3β-HSD, compared to that of control fed entire male pigs (Table 9). Furthermore the activities of CYP1A, 2A and 2C were larger in female pigs, compared to male. For the remaining part of the investigated mRNA, protein expressions and activities, no differences were observed.

5.0 Discussion

The influence of chicory root on regulation of porcine hepatic Phase I and II enzymes was investigated both *in vivo* and *in vitro*. This was done in order to evaluate the usefulness of chicory root to enhance the hepatic Phase I and II metabolism and thereby decrease the occurrence of boar taint. As boar taint predominantly occurs in male pigs, it is interesting to compare the constitutive expression of Phase I enzymes between genders.

In addition, a more methodological study of the methods for preparation of microsomes was included.

5.1 Microsomes – comparison of two different preparation methods

The use of hepatic microsomes for studying Phase I and II metabolism is well implemented, however differences in preparation methods exist (Diaz and Squires 2000a; Nelson et al. 2001; Nicolau-Solano et al. 2006; Zamaratskaia and Zlabek 2009). This makes it difficult to compare results on activity and expression between studies. The general procedure for preparation of microsomes is homogenization of the liver sample followed by an initial centrifugation (approx. 10.000 *g*) to remove cell debris, nuclei and mitochondria etc. The resulting supernatant is then centrifuged at high-speed (approx. 100.000 *g*) to sediment the microsomes. Before use the microsomes are dissolved in appropriate buffer (Nelson et al. 2001). Alternative methods using calcium to make aggregates and thereby reducing the speed and time of the second centrifugation have been developed (Nicolau-Solano et al. 2006). Knowledge about how the preparation method affects the microsomes with respect to specific protein concentrations and activities is important for the comparison of data obtained by different research laboratories.

The results presented in Paper I shows that CYP concentrations and activities in the microsomes are affected by the speed of the second centrifugation, with ultracentrifugation resulting in higher CYP concentrations and activities than low-speed combined with calcium aggregation. With respect to protein concentrations CYP2A6 was the only isoform being significantly different, while CYP1A2 and 2E1 almost reached significant levels. For the investigated CYP dependent activities, microsomes prepared by ultracentrifugation showed higher activities than microsomes prepared by low-speed centrifugation, apart from the CYP2A dependent coumarin hydroxylation. This discrepancy between CYP2A protein concentration and activity could be due to unspecific binding of the used human CYP2A6 antibody to other CYP isoforms or the contribution of other isoforms to the hydroxylation of coumarin.

The results presented in Paper I shows that comparison of data on CYP activities obtained using different microsome preparation methods should be made with caution. Due to the higher concentrations of CYPs and their activities in microsomes prepared by ultracentrifugation, this was the preparation method used for the rest of the experiments.

5.2 *In vivo* effects of chicory root on Phase I and II enzymes

The inclusion of chicory root in diets fed to pigs has shown to affect the microbial population in the intestine and to lower the concentration of skatole in fat. The study presented in Paper III-V was conducted to investigate the effect of dietary chicory root on hepatic Phase I and II enzymes. The investigated enzymes are all important for the catabolism of skatole and androstenone. In pigs, low levels of chicory in the feed (≤ 10 %) have been shown to decrease skatole concentration in faeces and fat (Lanthier et al. 2006; Rideout et al. 2004). Even though a previous study used higher amounts of chicory to show an effect on skatole concentrations in fat from sexually mature male pigs (Byrne et al. 2008), it was decided to use 10 % chicory in the diet to mimic a more realistic scenario from a production point of view.

The results presented in Paper III-V shows that inclusion of chicory in the diet affects Phase I enzymes on all "omic" levels, while no effect was seen on the investigated Phase II enzyme.

mRNA expression

The mRNA expression of CYP1A2 and 2A19 was highly increased in chicory fed pigs, while smaller increases were seen for CYP2D25, 2E1 and 3A29. Additionally the mRNA expression of 3β-HSD was increased in the chicory fed pigs. The only investigated CYP isoform not affected by chicory feeding was 2C33. Likewise the mRNA expression of SULT2A1 was not affected. Taken together the results indicate that the activation of AhR, CAR and PXR was increased in chicory fed pigs, although this was not the outcome of increased XR mRNA expression. If increased XR activation is the event behind the increased CYP expression in chicory fed pigs, it cannot be concluded from the present studies, which XRs that were activated, due to the fact that co-regulation of the different CYPs exists. Activation of XRs can be achieved in several ways. XRs can be activated by direct binding of the ligands, initiating translocation of the receptor to the nucleus, and gene transcription. For human and rodent XRs, ligand independent activation has been shown (Li and Wang 2010). Usually the XRs are retained in the cytosol in complex with chaperones. In ligand independent XR activation, the XR-chaperone-complex is disrupted by the

presence of an activation signal, leaving the XRs free and ready to enter the nucleus. Alternatively, the activities of the XRs could have been suppressed in the control pigs, meaning that some compounds in chicory have removed this suppression, either by direct or indirect interactions. In mouse, androstenol has been shown to act as an inverse agonist for CAR (Forman et al. 1998). If inverse agonists exist for all XRs and if they interact with porcine XRs in sexually mature male pigs needs to be investigated further. Noticeable, mRNA stabilisation and reduced turnover rates can also result in increased mRNA expression levels.

The unchanged SULT2A1 expression seems less surprising given the fact that the activities of Phase I metabolising enzymes is usually the rate limiting process in the elimination of xenobiotics (Parkinson 2001). A study by Sinclair *et al.* (2006) showed negative correlation between the hepatic SULT2A1 activity and androstenone concentrations in fat, suggesting that the activity of this Phase II metabolising enzyme is important. In contrast it has been shown that a reduction in testicular steroids, like testosterone, estradiol and androstenone, obtained by either surgical or immunocastration has no effect on mRNA and protein expression of SULT2A1 (Rasmussen et al. 2012). In accordance, SULT2B1 protein expression have been shown to be larger in high weight (115 kg) pigs, simultaneous with larger concentrations of testosterone, estradiol and androstenone, compared to low weight (90 kg) pigs (Zamaratskaia et al. 2012a).

Protein expression

Of the investigated proteins, increased expression of CYP1A2, 2A and 3β-HSD in chicory fed pigs was shown. These results correspond with the increases shown in mRNA expression. From the analysis of mRNA expression it could have been expected that an increase in the protein expression of CYP2E1 was present in chicory fed pigs. However, this was not the case. Discrepancies between hepatic CYP2E1 mRNA and protein expression in pigs have been observed before in a study by Brunius *et al.* (2012), investigating the simultaneous expression of mRNA and protein of several CYPs in entire and castrated male pigs. It was shown that castrated pigs had increased CYP2E1 mRNA expression, but unchanged protein expression, compared to entire male pigs. In fact post-transcriptional events have been shown to be important for regulation of CYP2E1 protein expression in rodents (Kocarek et al. 2000).

CYP dependent activity

In accordance with the observed increase in mRNA and protein expression of CYP1A2 and 2A19, increased CYP1A2 and 2A dependent activities were shown in chicory feed pigs. Likewise in accordance with the observed increase in CYP3A29 mRNA, increased CYP3A dependent activity was found in chicory fed pigs. For CYP2C and 2E1 dependent activity no difference was observed between chicory fed and control pigs. This seems less surprising given the fact that no difference was observed in CYP2C mRNA expression and CYP2E1 protein expression.

It should be noticed that the specificity of the substrates used to determine the activity of the individual CYPs is debated. The specificity of *p*-nitrophenol as a substrate for CYP2E1 has been questioned, as inhibition of CYP2A with polyclonal human antibodies was shown to inhibit *p*-nitrophenol hydroxylation as well (Skaanild and Friis 2007). Likewise, the specificities for the substrates used to determine porcine CYP1A2, 2C and 3A activity is as yet not fully established (Skaanild and Friis 2008; Zamaratskaia and Zlabek 2009; Zlabek and Zamaratskaia 2012). Nevertheless, these substrates are commonly used to determine specific CYP activities in both pigs and humans, and are believed to be primarily metabolised by the desired CYP isoform.

5.3 *In vitro* effect of chicory root on Phase I enzymes

The use of cell cultures to study gene regulation is a common approach. In the study presented in Paper VI the impact of chicory on gene regulation was investigated using primary porcine hepatocytes. At Department of Food Science, Aarhus University, the isolation and use of primary porcine hepatocytes was not implemented at the beginning of the present PhD project. In order to evaluate the impact of cryopreservation, the effect of time in culture on CYP mRNA expression in freshly isolated hepatocytes was compared to that of cryopreserved. As there was no difference in morphology and CYP expression between the fresh and cryopreserved hepatocytes with time in culture, it is suggested that cryopreserved hepatocytes can be used to study gene regulation.

To evaluate the ability and the magnitude by which the hepatocytes is able to modify CYP mRNA expression, their response to treatment with standard CYP inducers as well as pure secondary plant metabolites was determined. For induction of CYP1A2, the AhR ligand β-naphthoflavone was used, while for activation of CAR, CITCO and TCPOBOP were used. CITCO is a ligand of human CAR, while TCPOBOP is a ligand for murine CAR (Maglich et al. 2003; Moore et al. 2000; Tolson and Wang 2010). Dexamethasone treatment has been shown to induce multiple CYPs, including PXR regulated CYPs (Moore et al. 2000; Pascussi et al. 2000). Thus to

activate PXR, dexamethasone was used. As expected the hepatocytes increased the expression of CYP1A2 by treatment with β-naphthoflavone. Likewise, the expression of CYP3A29 was increased by treatment with dexamethasone. Surprisingly the mRNA expression of CYP2A19 was not increased by treatment with CITCO, while the lack of effect of TCPOBOP was expected (Gillberg et al. 2006). Chicory contains both sesquiterpene lactones and coumarines (Bais and Ravishankar 2001). Thus, in order to further validate the model, the hepatocytes were treated with a sesquiterpene lactone (artemisinin) and a coumarine (scoparone). Artemisinin increased the expression of CYP1A2, 2C33, 2D25 and 3A29, while scoparone increased the expression of CYP1A2 and 2D25. It shows that the porcine hepatocyte model is sensitive to secondary plant metabolites and that several CYPs can be induced at the same time.

The original rationale for initiating the *in vitro* experiment was to identify bioactive compounds present in chicory roots, acting as activators of the XRs and CYP inducers. However, as presented in Paper VI, large concentrations of chicory have a down-regulating effect on CYP expression, in conflict to the *in vivo* result presented in Paper III and V. The impact of chicory root on CYP mRNA expression was determined by the use of a methanolic extract of chicory root. After 24 hours of extraction, the methanolic phase was evaporated under vacuum, resulting in a 50-time reduction in the initial volume of the extract. The extract was then presented to the hepatocytes in a serial dilution, resulting in down-regulation of the mRNA expression of CYP1A2, 2C33, 2D25 and 3A29. However the use of plant extracts in *in vitro* models introduces some biases. When choosing a suitable solvent for extraction, it is often based on the assumption that a group of bioactive compounds are present in the plant material and that they possess the desired effect. Given the results presented in Paper VI on artemisinin treatment and studies by Burk *et al* (2005) and Simonsson *et al* (2006), as well as their presence in chicory roots (Bais and Ravishankar 2001; Cavin et al. 2005), sesquiterpene lactones were suggested as one of the candidates to induce CYP expression *in vivo*. As a methanolic extract of chicory root has been shown to contain sesquiterpene lactones (Cavin et al. 2005), methanol was chosen as the solvent. This however excludes compounds not soluble in methanol, which might possess CYP inducing properties. This could explain the contradicting *in vivo* and *in vitro* results.

Moreover, the discrepancies between the *in vivo* and *in vitro* effect of chicory root, can be explained by modification of the bioactive compound(s) during absorption in the gastrointestinal tract, before reaching the liver. CYPs are broadly distributed in the extrahepatic tissue, including the enterocytes of the gastrointestinal tract (Bader et al. 2000; Hansen et al. 2000;

Puccinelli et al. 2010). It has been shown that Phase I metabolites produced by one CYP can act as inducers of other CYPs (Gerbal-Chaloin et al. 2006). If such a metabolite is produced in the gastrointestinal tract during absorption and it can explain the discrepancies between the *in vivo* and *in vitro* effect of chicory root remains to be investigated.

Apart from that, other possible explanations can be more complex events like chicory induced changes in steroid levels affecting CYP expression, which might take place *in vivo*. This explanation is supported by the shown *in vivo* reduction in androstenone accumulation with chicory feeding.

Moreover, the contradictory results obtained *in vivo* using sexually mature male pigs compared to the *in vitro* results obtained in sexually immature female pigs, can be caused by the difference in plasma steroid profile of the animals. Nicolau-Solano and Doran (2008) investigated the effect of steroids on 3β-HSD expression in porcine hepatocytes isolated from two weight/age groups of male pigs. The endogenous steroid concentrations of the two groups of pigs were not measured, however, larger concentrations can be expected in the heavier/older pigs, according to Zamaratskaia *et al* (2004). The experiment showed differences in the effect of steroids on 3β-HSD expression between the two groups of pigs. This suggests that regulation of gene expression might dependent on the steroid profile of the pigs and that these differences still exist in the isolated hepatocytes. Thus, the difference in plasma steroid levels between the animals used for the *in vivo* and *in vitro* experiment, might explain the difference in response to treatment with chicory.

The results of treatment with dexamethasone, artemisinin and chicory extract in the *in vitro* experiment showed changes in the same CYPs, even though both up- and down-regulation was observed. This suggests that this group of CYPs can be regulated by a common pathway. What this pathway consists of and how it is regulated is still to be elucidated.

5.4 Gender-related differences in Phase I enzymes

Due to the nature of skatole production, it might seem unexpected that tainted meat almost exclusively occurs in male pigs. With that in mind the difference between gender in constitutive expression and activity of hepatic Phase I enzymes was investigated in the study presented in Paper II and V. Due to the production site of androstenone, it is noticeable that the presence of androstenone is missing in female pigs, and thereby the contribution of androstenone to the off-odour.

It is generally believed that the presence of steroids originating from the testis can explain the occurrence of tainted meat from sexually mature male pigs, partly by their impact on CYP regulation. However, other events like dimorphic secretion patterns of growth hormone have also been shown to be of importance for gender-related differences in expression of hepatic drug metabolizing enzymes (Waxman and Holloway 2009).

The constitutive mRNA expression of CYP1A2, 2A19, 3A29 and 3β-HSD was higher in female pigs, compared to entire male pigs. Apart from that no other differences in mRNA expression was observed. The differences in mRNA expression suggest differences in XR expression between genders. However, this was probably not the case, as no differences in the mRNA expression of AhR, CAR and PXR was observed. The larger expression of CYP1A2 and 2A19 was surprisingly not accompanied by a simultaneous larger protein expression. This observation seems even more surprising given the larger activity of CYP1A2 and 2A found in microsomes from female pigs, compared to that of male pigs. This indicates that, when comparing genders, the activity of Phase I enzymes are not strictly the results of protein expression, which again is not strictly regulated by the expression of mRNA. This is further supported by the observation that female pigs had larger mRNA expression of CYP3A29; while no differences in mRNA expression of CYP2C33 was seen. When investigating the activity of these CYP isoforms it was shown that female pigs had larger activity of CYP2C33; while no difference was observed for CYP3A29. It is worth noticing that the investigated isoforms do not fully represent all porcine CYP3A and 2C isoforms (Puccinelli et al. 2011). This might explain some of the observed discrepancy between mRNA expression and activity.

Taken together the results presented in Paper II and V indicate that female pigs have higher expression and activities of Phase I enzymes and thereby greater skatole catabolism compared to entire male pigs. This might explain the limited occurrence of tainted meat from female pigs (Bonneau 1982). The shown gender-related differences are in accordance with previous observations (Kojima et al. 2008; Skaanild and Friis 1999; Zamaratskaia et al. 2006). Given the results on CYP expression in castrated vs. entire male pigs (Brunius et al. 2012; Gillberg et al. 2006; Kojima et al. 2008; Whittington et al. 2004) and *in vitro* studies (Chen et al. 2008; Doran et al. 2002) it can be suggested that the concentration of endogenous steroids is important for the expression of the CYPs, in that large concentrations of steroids suppress CYP expression. Meaning that some steroids or their metabolites act as inverse agonists of the XRs. In fact it has been shown

using gene reporter assays that androstenone as well as its metabolites decreases porcine CAR activity (Gray et al. 2009).

Greater CYP expression and activity in female pigs compared to entire males, is not always the case. Zlabek and Zamaratskaia (2012) showed larger CYP3A dependent activity in microsomes from male pigs compared to female pigs. These findings are further supported by a study investigating PXR activity, showing that steroids like testosterone, estradiol and 3α-androstenol increased the activity of porcine PXR (Gray et al. 2010). However, manipulation with endogenous steroid levels by castration was shown to have no effect on CYP2C and 3A mRNA expression and activity (Tomankova et al. 2012).

5.5 Implication for boar taint

The project was initiated to investigate the impact of chicory root on hepatic Phase I and II metabolising enzymes, and thereby the influence of chicory on boar taint. As discussed above, the effect of chicory root varies between *in vivo* and *in vitro* experiments. Although the contradicting results, some reflections of the effect of chicory on boar taint can be put forward.

With the shown *in vivo* effects of chicory on skatole metabolizing enzymes, lower skatole levels can be expected in the chicory fed pigs. This was not the case, as no significant differences were found between the concentrations of skatole in chicory and control fed pigs. One possible explanation for this outcome might be that the skatole production and/or absorption was low in all pigs before the change in diet, and for that reason chicory could not suppress skatole concentrations more. Irrespectively of feeding regime all pigs had skatole concentrations below the threshold value (0.25 µg skatole/mg fat (Andresen 2006)). Another explanation for the lack of effect on skatole concentration might be that the activity of the CYPs was not increased sufficiently to decrease the skatole levels in the chicory fed pigs. Unfortunately, skatole levels were not determined in the female pigs, but it can be assumed that levels were below the threshold value, which is usually the case (Bonneau et al. 1992). Low skatole concentrations in female pigs are probably caused by sufficiently high hepatic skatole clearance. If then the CYP dependent activity is compared between the chicory fed pigs and the female pigs, the chicory fed pigs had similar or even greater CYP activity that the female pigs. This suggests that the chicory induced increase in CYP activity is sufficient to keep skatole levels below the threshold value.

Another important compound for the sensory perception of pig meat is androstenone. In the study present in Paper IV it was shown that the chicory fed pigs, had lower androstenone

concentrations in fat compared to the control fed pigs. However, in both groups of pigs, individuals with androstenone concentrations above the threshold value (1 µg androstenone/mg fat (Andresen 2006)) were observed. For the control fed pigs, 67 % of the pigs had androstenone concentrations above the threshold value, while the corresponding number for the chicory fed pigs was 21 %. As an increased mRNA and protein expression of the androstenone metabolizing enzyme 3β-HSD was observed the reduced androstenone concentrations found in the chicory fed pigs might be explained by increased hepatic androstenone clearance. Additionally, the decreased androstenone levels in the chicory fed pigs might explain the increased CYP expression observed in the same pigs.

The reduced androstenone levels found with chicory feeding might imply that the anabolic potential of the pigs was reduced, which might affect the benefits of producing entire male pigs. This was not the case, as no difference in plasma testosterone and IGF-1 concentrations was observed between chicory and control fed pigs. Estradiol is, in contrast to other species, found in large concentrations in entire male pigs, where it contributes to the anabolic potential of the animal (Claus et al. 1994). In the chicory fed pigs a tendency ($p = 0.06$) towards lower estradiol concentrations in plasma was observed. This might indicate that the anabolic potential of the chicory fed pigs, was decreased compared to control fed pigs. This however didn't affect carcass weight as no difference between chicory and control fed pigs could be observed.

6.0 Conclusions

When studying hepatic Phase I and II metabolism, the chosen method is often very important. In the present PhD thesis a protocol for isolation of primary porcine hepatocytes is included, as well as a comparison of two different methods for microsome preparation. It was shown that CYP expression and more importantly activities is greatly affected by the used method for microsome preparation. Using ultracentrifugation compared to low-speed centrifugation combined with calcium aggregation, results in greater microsomal CYP expression and activity. This shows that the preparation method is important when comparing results from different research labs.

The present PhD study, aims to investigate the impact of chicory on hepatic Phase I and II metabolism, in relation to boar taint. The obtained results show for the first time that dietary administrated chicory root modifies hepatic CYP expression and activity.

Feeding sexually mature male pigs with dried chicory root for 16 days was shown to up-regulate the mRNA expression of CYP1A2, 2A19, 2D25, 2E1 and 3A29 mRNA, as well as CYP1A2 and 2A protein expression. This up-regulation gave rise to increased CYP1A2, 2A, and 3A dependent enzyme activity. As these CYPs are involved in the metabolism of skatole, decreased skatole accumulation was expected in the chicory fed pigs. This was not the case, as no difference between the chicory and control fed pigs was observed. It should be noticed that the levels of skatole were well below the acknowledged threshold value, for sensory perception of boar taint, in both groups. Likewise, no differences in testosterone, IGF-1 and estradiol levels were observed. The chicory fed pigs showed decreased accumulation of androstenone in fat. As the expression of the hepatic androstenone metabolising enzyme 3β-HSD was up-regulated in the chicory fed pigs, it is concluded that androstenone metabolism is increased by chicory feeding. The lower androstenone concentration found with chicory feeding might explain the increase in hepatic CYPs as well. Taken together the results shows that, chicory feeding increase the hepatic expression of Phase I enzymes without affecting the anabolic potential of the pigs and that chicory feeding can be used to lower the occurrence of boar taint.

In contrast to the observed *in vivo* effects of chicory root, *in vitro* experiments using primary porcine hepatocytes showed opposite results. By treating hepatocytes with various concentrations of a methanolic extract of dried chicory root it was shown that mRNA expression of CYP1A2, 2C33, 2D25 and 3A29 was down-regulated. Additionally, it was shown that the standard CYP inducers, β-naphthoflavone and dexamethasone, increased the mRNA expression of CYP1A2 and 3A29. Likewise, artemisinin and scoparone increased the mRNA expression of CYP1A2,

2C33, 2D25 and 3A29. What causes the contradicting results obtained from the *in vivo* and *in vitro* experiments needs further investigation.

A gender-related difference in hepatic CYPs was shown comparing female pigs vs. entire male pigs. Female pigs showed greater constitutive mRNA expression of CYP1A2, 2A19 and 3A29, as well as CYP1A2, 2A and 2C dependent enzyme activity. There were no observed differences in CYP protein expression between the two genders. The observed CYP activities in chicory fed pigs were comparable to those found in female pigs. This suggests that chicory can increase CYP activities to sufficiently high levels, to lower skatole concentrations.

7.0 Perspectives for future research

The outcome of this project is not only of interest to the future research on boar taint, but also to the research about dietary influence on hepatic detoxification in general – in both humans and animals.

The impact of absorption on hepatic CYP induction

The results presented in this PhD thesis on the impact of chicory on CYP expression and activities shows contradicting results *in vivo* and *in vitro*. This, as discussed in the *Discussion*, can be due to modification of the compounds found in chicory during absorption, e.g. by reactions in the intestinal epithelium. So to better understand the impact of chicory *in vivo*, the mechanism of gastrointestinal absorption needs further investigation.

Moreover, the *in vivo* results needs to be confirmed in both genders, and the impact of exposure time and dose are likewise important parameters to be investigated. In the literature there are studies showing both effects and no effects on boar taint compound by feeding with chicory. To further elucidate the effect of chicory on skatole and androstenone concentrations in adipose tissue of sexually mature male pigs, it could be of importance to make feeding trials in pigs genetically disposed to have high concentrations of boar taint compounds.

The effect of gender on CYP regulation

An alternative explanation for the contradicting *in vivo* and *in vitro* results are factors like age and gender of the used animals, which possibly affect the hormone load of the animals, before the exposure to chicory. Thus, the significance of age and gender in regulation of porcine CYPs needs to be further elucidated. Likewise, the effect of "pre-programming" by steroids can add new knowledge about CYP regulation and the impact of dietary components.

Function of porcine XRs and CYP induction

In the literature there are numerous examples on species specific differences in XR ligands and their impact on CYP induction. With respect to, porcine XRs and their ligands some studies have investigated CAR and PXR in details, while data on porcine AhR and other XRs are scarce or missing. In order to fully use dietary compounds or other substances to manipulate with the constitutive expression of porcine CYPs, detailed knowledge about their regulation is essential. This knowledge could also serve to further validate/evaluate the usefulness of pigs as models for humans.

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Paper I

Comparison of Cytochrome P450 concentrations and metabolic activities in porcine hepatic microsomes prepared with two different methods

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Comparison of cytochrome P450 concentrations and metabolic activities in porcine hepatic microsomes prepared with two different methods

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ABSTRACT

In the present study, porcine liver microsomes prepared by a conventional ultracentrifugation method were compared with microsomes prepared by a calcium aggregation method. Protein concentrations and activities of several cytochrome P450 enzymes were measured. It was concluded that using a calcium aggregation method for microsome preparation resulted in lower activities of porcine 7-ethoxyresorufin *O*-deethylase (EROD), 7-methoxyresorufin *O*-demethylase (MROD), 7-pentoxyresorufin *O*-dependentylase (PROD) and *p*-nitrophenol hydroxylase (PNPH), compared to ultracentrifugation. Protein concentrations of CYP1A2 and CYP2E1, measured by Western blot, were similar in the microsomes prepared by the two methods, whereas CYP2A protein concentrations were significantly lower in the microsomes prepared by the calcium aggregation method. The choice of homogenization buffer (TRIS with addition of either 250 mM sucrose or 2 mM EDTA) did not affect either individual CYP450 protein concentration or the rates of CYP450-mediated reactions. Freeze/thawing of microsomes did not affect the activities of EROD, MROD, COH and PNPH in the microsomes, indicating the stability of the measured isoforms following three cycles of freezing/thawing. A reduction in the activity of PROD was observed after the third freeze/thawing cycles of the microsomes prepared by both methods.

1. Introduction

In vitro methods play an essential role in toxicological studies. Microsomes are widely used for *in vitro* investigation of the metabolic profile of various exogenous and endogenous compounds, as well as cytochrome P450 (CYP) expression and activity (Asha and Vidyavatha, 2010). Microsomes are easy to use and relatively stable as they can be stored at −80 °C for several years without a decrease in catalytic activity of the CYP enzymes (Yamazaki et al., 1997). However, conventional preparation of microsomes by high-speed centrifugation is not always convenient because of the need of expensive centrifuges and time-consuming procedures. Additionally, this method requires a large amount of sample material. A calcium aggregation method using low speed centrifugation was demonstrated to be an alternative method to prepare microsomes from the liver of guinea pig, rat, mouse and rabbit (Ecobichon, 1976; Walawalkar et al., 2006) and from the brain of rat and mouse (Ravindranath and Anandatheerthavarada, 1990). However, only limited data on the suitability of this method with respect to porcine tissues are available. Nicolau-Solano et al. (2006) used the porcine liver microsomes prepared by the calcium aggregation method to characterize the activity of hepatic 3ß-hydroxysteroid dehydrogenase (3ß-HSD). A slightly modified method to prepare microsomes from porcine liver were used to measure activities of 7-ethoxyresorufin *O*-deethylase (EROD) and 7-methoxyresorufin *O*-demethylase (MROD) (Zamaratskaia and Zlabek, 2009) as well as 7-pentoxyresorufin *O*-dependentylase (PROD), coumarin hydroxylase (COH) and *p*-nitrophenol hydroxylase (PNPH) (Zamaratskaia et al., 2009). However, the potential inhibition of enzymes by calcium might be a severe limitation to the use of the calcium aggregation method (Guarnieri et al., 1976; Ilyina et al., 2003). Thus, a more extensive validation of this method with regard to protein concentration, activity and stability in pigs is needed.

In the present study, protein concentration of CYP1A2, CYP2A and CYP2E1 as well as the activities of EROD, MROD, PROD, COH and PNPH in porcine microsomes prepared by high-speed centrifugation and the calcium aggregation method have been compared. Study of those CYP450 isoforms is particularly relevant, since they metabolize various endogenous compounds and pollutant organic xenobiotics in many species. Additionally, we investigated the effect of homogenizing buffer on the above-mentioned parameters, and the effects of cycles of freezing/thawing on catalytic activities of the CYP enzymes.

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2. Material and methods

2.1. Animals and sampling

Liver samples (approximately 50 g) from freshly eviscerated liver of 6 pigs (3 males and 3 females) of a crossbred between Landrace × Yorkshire dam and Duroc boars (age 164 days) were collected, immediately frozen in liquid nitrogen and transported to the laboratory. Then, the samples were stored at −80 °C until used for microsome preparation.

2.2. Microsome preparation

Approximately 6 g liver tissue were minced in Tris–sucrose buffer (10 mM Tris–HCl, 250 mM sucrose, pH 7.4) or Tris–EDTA buffer (50 mM Tris–HCl, 150 mM Potassium chloride, 2 mM EDTA, pH 7.4) in a ratio of 1:2 (weight/volume) and homogenized (Polytron 2100, setting 22) for 2 × 20 s with a 10 s break between. After 10 min centrifugation (Eppendorf Centrifuge 5417R) at 10.000×*g*, 4 °C, the supernatant (crude homogenate) was divided into two and microsomes were prepared either using ultracentrifugation or low speed centrifugation and Ca^{2+} aggregation. All steps were carried out on ice.

For ultracentrifugation, crude homogenate was diluted in appropriate buffer (Tris–sucrose or TRIS–EDTA) to a final volume of approximately 25 ml and centrifuged (Beckman Coulter Optima™L-80 XP Ultracentrifuge) at 100,000×*g* for 60 min at 4 °C (Diaz and Squires, 2000; Rasmussen et al., 2010). For Ca^{2+} aggregation, crude homogenate was diluted with appropriate buffer (TRIS–sucrose or TRIS–EDTA) containing 8 mM $CaCl_2$ to a final volume of approximately 25 ml. After 4 min of incubation the samples were centrifuged (Beckman Coulter Optima™L-80 XP Ultracentrifuge) at 25,000×*g* for 30 min at 4 °C (Zamaratskaia and Zlabek, 2009).

The cytosolic fractions obtained from both methods were retained and the microsomal pellets were suspended in a buffer containing 50 mM Tris–HCl, 10 mM KH_2PO_4, 0.1 mM EDTA, 20% glycerol (pH 7.4) and stored at −80 °C for later enzymatic assays.

2.3. Total protein content

Protein concentrations in the microsomal and cytosolic fractions were assayed with two methods. Total protein concentration was measured with two commercially available kits, Bio-Rad protein assay kit, (Bio-Rad laboratories Inc., Hercules, CA, USA) or the Pierce® BCA protein assay kit (Thermo scientific, Rockford, IL, USA). Both assays were performed according to manufacturer's instructions with bovine serum albumin as standard.

2.4. Enzymatic assays

The activities of EROD, MROD, PROD, COH and PNPH in porcine microsomes were measured as previously described (Zamaratskaia et al., 2009). The same assays were performed in the cytosolic fraction. All measurements were performed in duplicate. To express enzymatic activities per mg of protein, total protein concentrations obtained by the Pierce® BCA protein assay kit method were used.

2.5. Western blotting

Liver microsomes were dissolved in TS-buffer (10 mM Tris–base, 0.9% NaCl, pH 7.4) and equal protein amounts were separated by electrophoresis using a SDS–polyacrylamide gel (12% Tris–Bis (Bio-Rad), 200 V for approximately 1 h). After electroblotting (150 mA for 1½ h) onto a polyvinylidene difluoride membrane (Bio-Rad) and blocking with 2% Tween 20 in TBS-buffer (50 mM Tris–base, 0.5 M NaCl, pH 7.4) for 5 min, the membrane was incubated with primary antibody (polyclonal anti-human CYP1A2, 2A6 and 2E1 diluted in TBS-buffer with 0.1% Tween 20) over night at 4 °C. Afterwards the membrane was washed 2 × 10 min in TBS-buffer with 0.1% Tween 20 and incubated with Alexaflour448 attached secondary antibody (DAKO). After 6 × 5 min washing with TBS-buffer with 0.1% Tween 20, the relative protein concentration was visualized by scanning on a Molecular Imager® FX (Bio-Rad) scanner and quantified with Quantity One Version 4.5.2 (Bio-Rad).

2.6. Freezing and thawing of porcine microsomes

Prepared microsomes were frozen in two stocks and kept at −80 °C until needed. Stock 1 was later used for measurements of protein concentrations, while stock 2 was used for analysing enzymatic activities. Prior to the first set of analysis, the microsomes were thawed on ice and analysed for total protein concentration. At the same day, the microsomes were diluted with ice-cold resuspension buffer (0.1 M potassium phosphate buffer, pH 7.4, containing 25% glycerol) to achieve a final concentration of 4 mg protein/ml, and the assays for the activities of all enzymes (Thaw 1) were performed. The remaining microsomes were divided into five stocks; one for each of the five activity measurements (200 µl in each 1.5 ml Eppendorf tube) and then refrozen (overnight at −80 °C). The next day, the samples were thawed (Thaw 2), 100 µl of the microsomes were taken from each Eppendorf tube and used for enzymatic assays. The remaining microsomes (100 µl in each 1.5 Eppendorf tube) were refrozen at −80 °C and used for enzymatic assay the next day (Thaw 3).

2.7. Statistical analysis

For Western blotting all samples to be compared were run on the same gel. Due to corresponding molecular weight, the different CYP isoforms were separated on different gels. Obtained data were expressed relative to the average of the values for microsomes prepared by calcium aggregation using Tris–sucrose buffer.

Data were evaluated using SAS (version 9.1. SAS Institute, Cary, NC, USA). To test for normal distribution of the measured variables the Kolmogorov–Smirnov test was used. Since the observed values did not show significant deviation from normal distribution, no further data transformation was used. Fixed effect of microsome preparation method (ultracentrifugation vs Ca-aggregation method), homogenization buffer (Tris–sucrose vs Tris–EDTA) and interactions on measured parameters were evaluated with the procedure GLM. Procedure GLM was used to calculate partial correlations between total protein concentrations measured by the two methods (BCA vs Bio-Rad). Fixed effect of freeze/thawing on enzymatic activities was evaluated with procedure GLM. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Total protein concentrations in the hepatic microsomes

The partial correlation coefficient between the total protein concentrations in the hepatic microsomes obtained by the two methods was 0.636 ($p < 0.01$). The mean value and standard deviation (SD) for the BCA method were 12.7 mg/ml (SD 4.40) and for Bio-Rad 12.6 mg/ml (SD 5.35).

Total protein recovery by the two methods and buffers was estimated from the obtained sample volumes and protein concentrations. Of the total protein amount in the crude homogenate 10.8% and 11.3% were recovered by the calcium aggregation meth-

od with Tris–sucrose and Tris–EDTA buffer, respectively. For the ultracentrifugation method the same values were 8.9% and 8.8%, respectively. There was significant (the overall standard error of the data was 1.33%; $p < 0.05$) difference between the two methods but not buffers.

3.2. Activities of hepatic cytochrome P450

Activities of the respective enzymes in the microsomes prepared using Tris–sucrose or Tris–EDTA buffer showed no significant differences ($p > 0.05$; Table 1). The activities were higher in the microsomes prepared by ultracentrifugation ($p < 0.05$) except for the COH activities which were similar in the microsomes prepared by the two methods ($p = 0.334$; Table 1).

Only trace activities of EROD (from 0.9 to 1.9 pmol/min/mg) and COH (from 0.1 to 1.2 pmol/min/mg) were detected in the cytosol fractions origin from both methods, compared to the activity measured in the microsomes. PROD, MROD and PNPH activity were not detectable.

3.3. Western blot analyses on hepatic cytochromes P450

All used antibodies detected one single bands corresponding in Mw to CYP450 (Fig. 1). Protein bands in the microsomes prepared by the ultracentrifugation method showed higher protein concentrations, compared to the calcium aggregation method. However, the statistical analyses revealed that only the concentration of CYP2A was significantly affected by the method used for microsome preparation. Concentrations of CYP1A2 and CYP2E1 were slightly higher in the microsomes prepared by ultracentrifugation ($p = 0.065$ and 0.087 for CYP1A2 and CYP2E1, respectively), but those differences were not statistically significant (Table 1) due to large variation in the constitutive CYP expression between animals.

3.4. Effect of freeze/thawing on activities of hepatic cytochrome P450

No difference in the response of CYP activities to freeze/thawing was observed between microsomes prepared by the ultracentrifugation and calcium aggregation methods ($p > 0.05$). Thus, the effect of freeze/thawing was statistically evaluated using combined data from the microsomes prepared by both methods. The repeated freezing/thawing cycles showed different effect on the investigated CYP enzymes activity. Freeze/thawing of microsomes had no affect on the activities of EROD, COH and PNPH (Table 2). Slight increase in MROD activity was observed in the microsomes after the third freeze/thawing. The activity of PROD was not altered by the two first freeze/thawing, but after the third freeze/thawing the activity was markedly reduced (Table 2). This reduction was observed in the microsomes prepared by both methods.

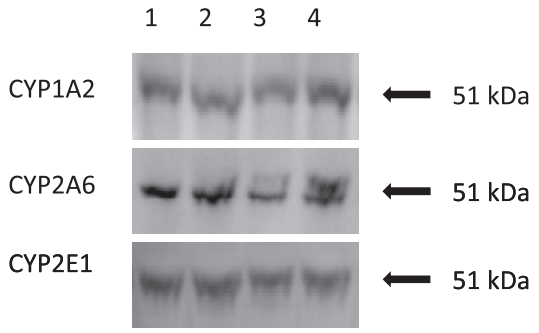

Fig. 1. Representative blots obtained by Western blotting of porcine microsomes prepared either by ultracentrifugation or the calcium aggregation method. The blots were only used once and probed with either antibodies against human CYP1A2, CYP2A6 or CYP2E1. Lane 1, calcium aggregation method with Tris–sucrose as homogenization buffer; Lane 2, ultracentrifugation method with Tris–sucrose as homogenization buffer; Lane 3, calcium aggregation method with Tris–EDTA as homogenization buffer; Lane 4, ultracentrifugation method with Tris–EDTA as homogenization buffer.

Table 2
Effect of freeze/thawing on the activities of cytochrome P450 enzymes in the microsomes.

	Thaw 1	Thaw 2	Thaw 3	Standard error	*p*-value
EROD, pmol/min/mg	123.5	116.5	126.5	7.23	0.610
MROD, pmol/min/mg	18.1	20.7	21.2	0.96	0.060
PROD, pmol/min/mg	6.4[a]	6.7[a]	4.6[b]	0.41	0.001
COH, pmol/min/mg	72.3	72.5	71.9	10.19	0.999
PNPH, pmol/min/mg	659.0	696.5	590.9	43.09	0.221

All data presented as least-squares means and standard errors. Enzyme activities in the microsomes prepared by both methods were included in this analysis as no differences in the response to freeze/thawing between the methods were observed ($p > 0.5$). Within a row, significantly different ($p < 0.05$) means are indicated by different superscripts. EROD, 7-ethoxyresorufin *O*-deethylase; MROD, 7-methoxyresorufin *O*-demethylase; PROD, 7-pentoxyresorufin *O*-dependentylase; COH, coumarin hydroxylase; PNPH, *p*-nitrophenol hydroxylase.

4. Discussion

The role of cytochrome P450 enzymes in drug metabolism in human is well known (Bertz and Granneman, 1997) and their mediated reactions are therefore widely studied. However, in such studies different methods for microsome preparation are often used as well as different protocols for enzyme activity assays. The studies on the effects of different methods on catalytic activities of CYP450s are usually performed on human microsomes (Nelson et al., 2001), while porcine microsomes have attracted

Table 1
Protein concentration and activities of cytochrome P450 enzymes in the microsomes prepared by two different methods.

Method	Ultracentrifugation		Ca-aggregation		Standard error	*p*-value method	*p*-value buffer	*p*-value buffer × method
Buffer	Tris–sucrose	Tris–EDTA	Tris–sucrose	Tris–EDTA				
CYP1A2 content, arbitrary units	138.0	154.2	100.0	111.7	20.60	0.065	0.505	0.914
CYP2A6 content, arbitrary units	126.6	124.4	100.0	99.7	11.38	0.036	0.914	0.934
CYP2E1 content, arbitrary units	122.5	133.4	100.0	110.9	12.49	0.087	0.394	0.999
EROD, pmol/min/mg	137.8	142.2	104.4	109.6	13.76	0.026	0.730	0.978
MROD, pmol/min/mg	21.3	19.6	15.1	16.3	1.72	0.012	0.892	0.416
PROD, pmol/min/mg	7.9	6.5	5.4	5.6	0.70	0.021	0.382	0.260
COH, pmol/min/mg	87.7	78.4	62.1	60.9	21.78	0.334	0.814	0.854
PNPH, pmol/min/mg	790.5	747.0	507.4	591.2	78.69	0.011	0.800	0.428

All data presented as least-squares means and standard errors. EROD, 7-ethoxyresorufin *O*-deethylase; MROD, 7-methoxyresorufin *O*-demethylase; PROD, 7-pentoxyresorufin *O*-dependentylase; COH, coumarin hydroxylase; PNPH, *p*-nitrophenol hydroxylase.

surprisingly little attention especially in the light of the usefulness of pigs as a liver donor species or the use in drug tests. Additionally, porcine microsomes are often used in the studies on the effects of industrial pollutants and pharmacologically active substances on CYP450s (Szotáková et al., 2004). Thus, it stresses the importance of further studies on CYP450s in porcine microsomes. However, differences in methods for microsome preparation might affect the final result and make it difficult to compare data on enzymatic activities in pigs obtained from different laboratories. Therefore, the critical factors in microsomes preparation should be identified. The novelty of this study mainly concerns the choice of species (pig) for investigation of those factors. To study hepatic metabolism in pigs is of particular importance as it will be gaining further insight into the advantages of the use of porcine microsomes for drug metabolism studies, especially in the context of the unlimited supply of pig liver tissue in comparison to human liver tissue.

In the present study, we focused on the comparison of two methods for preparing microsomes from porcine liver; one with and one without the use of ultracentrifugation. Additionally, the preparation of microsomes with the use of two different homogenization buffers, one with Tris–sucrose and one with Tris–EDTA, was compared. To our knowledge, this is the first study comparing two methods of microsomes preparation from porcine liver samples in terms of protein concentration and enzyme activities.

The results show that the two different methods for microsome preparation yield reasonably similar results regarding specific protein concentrations as demonstrated by Western blot analysis. However, the use of the calcium aggregation method to prepare microsomes resulted in an apparent higher protein recovery and a reduction of enzyme activity. Thus, it can be suggested that the differences in enzymatic activities might be due to the inhibition of those enzymes by calcium. However, great numerical differences in protein concentrations, which corresponded very well to the differences in enzyme activities, indicate that higher activities in the microsomes prepared by ultracentrifugation are due to higher content of the corresponding enzyme isoform. It is likely that the differences in protein concentration did not reach statistical significance because of large variations in constitutive expression within groups.

The composition of the homogenizing buffer is thought to be one of the most important variables in microsome preparation. Nelson et al. (2001) demonstrated that inclusion of sucrose in the homogenizing buffer is an important factor in microsome preparation regarding the relative specific activity in the microsomes, but it did not affect either protein content or enzyme activity in our study.

During the fractionation of liver homogenate, cross-contamination of prepared fractions, e.g. insufficient separation of microsomal and cytosolic protein, might affect the final results. Our study however, showed only traces activities of EROD and COH in the cytosol fractions. Activities of other measured enzymes were not detectable.

A few studies have investigated the effects of freezing and thawing on catalytic activities of CYP450 (Pearce et al., 1996; Yamazaki et al., 1997). Freeze/thawing of microsomes did not affect the activities of EROD, COH and PNPH in the microsomes, indicating the stability of the measured isoforms after at least three cycles of freezing/thawing. A reduction in the activity of PROD was observed after the third freeze/thawing cycles of the microsomes prepared by both methods. Thus, we recommend avoiding repeated freezing and thawing of microsomes for the measurement of PROD activities to obtain more accurate results.

5. Conclusions

Taken together, our findings for the first time suggest the following: (1) preparation of the microsomes from porcine liver using the ultracentrifuge results in higher amount of CYP2A protein and greater activities of EROD, MROD, PROD and PNPH compared to the calcium aggregation method; (2) homogenization in Tris buffers with addition of either 250 mM sucrose or 2 mM EDTA affected neither CYP450 protein isoforms concentration nor the rates of CYP450-mediated reactions.

Furthermore the difference in protein recovery and cytochrome activity/expression indicates differences in the composition of the microsomes. However this needs to be investigated further.

6. Conflict of interest

None declared.

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Paper II

Gender-related differences in cytochrome P450 in porcine liver – implication for activity, expression and inhibition by testicular steroids

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Gender-related Differences in Cytochrome P450 in Porcine Liver – Implication for Activity, Expression and Inhibition by Testicular Steroids

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Contents

In pigs, the hepatic cytochrome P450 (CYP) 1A2, 2A and 2E1 activity is important in the regulation of skatole accumulation in adipose tissue. This study investigated gender-related differences in CYP1A2, 2A and 2E1 dependent activity, protein and mRNA expression. This study also investigated the gonadal steroid dependent inhibition of CYP activity in relation to gender and dietary composition. Microsomes were prepared from the liver of female and entire male pigs (Landrace × Yorkshire sire and Duroc boars) reared under similar conditions and slaughtered at an age of 164 days. A group of entire male pigs fed dried chicory root for 16 days prior to slaughter were included in the study. CYP activities were assessed by the use of probe substrates, whilst mRNA and protein expression were analysed by RT-PCR and Western blotting. Furthermore inhibition of CYP dependent activity by gonadal steroids was assessed *in vitro*. Microsomes from female pigs had greater CYP1A2 and 2A activity, as well as mRNA expression compared to entire male pigs. The antibodies used did not detected differences in protein expression. *In vitro* inhibition by 17β-oestradiol, oestrone, androstenone and 3β-OH androstenol of CYP2E1 activity in microsomes from entire male pigs as well as inhibition of CYP1A activity in chicory fed entire male pigs was observed. Apart from that no effect of steroids was shown. In conclusion, female pigs show greater CYP activity and mRNA expression. Including chicory in the diet for 16 days changed the gonadal steroid dependent inhibition of CYP activity in entire male pigs.

Introduction

The cytochrome P450s are encoded by a superfamily of genes, and play a critical role in the bioactiviation and detoxification of xenobiotic substances (reviewed by Guengerich 2008). In porcine liver three isoforms of cytochrome P450 (CYP) (1A2, 2A, 2E1[1]) have been reported to be involved in the Phase I metabolism of skatole (Babol et al. 1998; Diaz and Squires 2000; Terner et al. 2006; Matal et al. 2009). Thus the activity of these enzymes is important concerning the regulation of skatole accumulation in adipose tissue. High concentrations of skatole in the adipose tissue are often associated with a faecal-like odour of the meat and are mainly found amongst entire (non-castrated) male pigs at the onset of puberty.

Gender-related differences in hepatic xenobiotic metabolism and gene expression have been extensively studied in human and rats, but also shown in other species (for reviews see Mugford and Kedderis 1998; Waxman and O'Conner 2006). In pigs expression and activity of the CYPs vary with genetic profile and hormonal status (reviewed by Zamaratskaia and Squires 2009), as well as gender, the latter both in mini- and conventional pigs (Skaanild and Friis 1999; Zamaratskaia et al. 2006). In the small sized breed Meishan pig, the constitutive expression and activity of CYP1A were greater in females and castrated male pig than in entire male pigs (Kojima et al. 2008). Zamaratskaia et al. (2006) found greater activity of CYP2A and CYP2E1 in female pigs compared to entire male pigs, although this difference was dependent on age. Furthermore, it was shown that surgical castration as well as immunocastration results in increased activities of CYP1A, CYP2A and CYP2E1 (Zamaratskaia et al. 2009). Using 50% Meishan pigs crossed with Large White, Whittington et al. (2004) showed higher CYP2E1 expression in surgically castrated male pig compared to entire males. Taken together, gender-related differences are thought to be due to the absence (in castrated pigs) or low levels (in female pigs) of gonadal steroids. Yet, it is still not clear if the differences in activity are caused by differences in protein expression and/or direct regulation of the catalytic activity.

One of the major factors regulating the catalytic activity of CYPs is testis derived steroids. An *in vitro* study using microsomes from entire male pigs showed that high concentrations of androstenone inhibited the formation of several skatole metabolites (Babol et al. 1999). Furthermore, using microsomes from male pigs, androstenone and 17β-oestradiol have been shown to inhibit the activity of CYP2E1 (Zamaratskaia et al. 2007). In a recent study we showed that androstenone and 17β-oestradiol in concentrations found in sexual mature male pigs decreases CYP2E1 activity by 30% in the microsomes from male pigs, whilst no effect was detected in the microsomes from female pigs (Rasmussen et al. 2010). The same study showed no effect of steroids on the activity of CYP2A. Results from a study of steroid dependent inhibition of CYP activity in microsomes from entire and surgically castrated pigs, showed a different response between the two groups of animals, indicating that the *in vivo* hormonal exposure of the pig may influence the response of specific CYPs to the *in vitro* treatment (Zamaratskaia et al. 2009). Thus, the results from previous studies suggest that regulation of CYP activities differs between genders within the same species. Yet, the existence of gender-related differences in steroid dependent regulation of the catalytical properties needs to be confirmed and the mechanism behind this elucidated (Zamaratskaia et al. 2007, 2009; Rasmussen et al. 2010).

[1]The nomenclature of cytochrome P450 usually consist of a 'CYP' followed by an Arabic number denoting the family, a letter denoting the subfamily and an Arabic number representing the individual gene within the subfamily.

It has been known for some time, that the activity of CYP1A2, 2A and 2E1 are important in regulating the concentration of skatole in fat and plasma of pigs (Matal et al. 2009; Zamaratskaia and Squires 2009). The relative importance of those enzymes in skatole metabolism is still discussed. Matal et al. (2009) suggested that CYP2A19 is more active in metabolizing skatole in pigs than CYP1A2 and CYP2E1. The superior role of CYP2A activity compared to CYP2E1 in skatole metabolism was also suggested by Zamaratskaia et al. (2005a). In contrast, Terner et al. (2006), using porcine hepatocytes, found that CYP2E1 was more active than CYP2A. Both, CYP2A and 2E1 are regulated by testicular steroids, as demonstrated by various *in vitro* and *in vivo* studies. The regulation of CYP2A and CYP2E1 by testicular steroids in pigs was suggested to be transcriptional (Tambyrajah et al. 2004; Gillberg et al. 2006). Additionally, testicular steroids can directly inhibit CYP2E1 activity (Zamaratskaia et al. 2007; Rasmussen et al. 2010).

Less is known about the involvement of testicular steroids in the regulation of CYP1A2 expression and activity in pigs. Kojima et al. (2008) used administration of testosterone propionate to study the effect of androgens on CYP1A in immature Meishan pigs. It was shown that pigs treated with testosterone propionate exhibit lower mRNA expression of CYP1A1 and CYP1A2. It is also known that castration of pigs significantly increases the 7-ethoxyresorufin deethylation activity (Zamaratskaia et al. 2009), a marker of CYP1A activity. In the same study it was shown that oestrone and androstenone inhibited metabolism of 7-ethoxyresorufin. It has previously been shown that inhibition of CYP2E1 in microsomes by androstenone and 17β-oestradiol only occurs if an additional step is included, that is pre-incubation with the steroid before adding the substrate (Rasmussen et al. 2010). It was suggested that a metabolite, which originates from the break-down of androstenone, was responsible for the inhibition of the enzyme activity. The primary metabolite of androstenone is 3β-OH androstenol (Brophy and Gower 1972; Doran et al. 2004; Sinclair et al. 2005). Yet it has never been investigated if this metabolite inhibits the activity of CYP2E1. In addition to 17β-oestradiol, entire male pigs posses high amounts of oestrone (Claus et al. 1989). The concentration of oestrone increases with age and is positively correlated to the concentration of skatole (Zamaratskaia et al. 2005b). Thus, a possible role of oestrone in regulating skatole metabolism as an inhibitor of skatole-metabolizing enzymes was suggested (Zamaratskaia et al. 2009).

The skatole concentration in plasma and back-fat has shown to be strongly affected by dietary composition (reviewed by Zamaratskaia and Squires 2009). Diets containing chicory root significantly reduces skatole levels in pigs (Hansen et al. 2006), which is believed to be mainly due to reduction of the intestinal skatole production. On the other side, dietary intake of fibres has shown to increase the expression and activity of CYP in rats and cows (Nugon-Baudon et al. 1996; Lemley et al. 2010). Thus it seems likely that skatole metabolism in the liver to some extent is affected by the dietary composition. At present it is not clear if dietary ingredients are able to change the catalytic properties of the CYPs in pigs.

The aim of the present study was to investigate gender-related differences in catalytic activities of CYP1A, CYP1A2, CYP2A and CYP2E1 in pigs and to analyse if the constitutive protein and mRNA expression of CYP1A2, CYP2A and CYP2E1 differs. Furthermore we investigated the inhibition of CYP1A, CYP1A2 and CYP2E1 activities by the steroids 17β-oestradiol, oestrone, androstenone and 3β-OH androstenol in microsomes prepared from entire male and female pigs. The choice of steroids for the *in vitro* studies was based on our results from a previous investigation (Rasmussen et al. 2010). In order to evaluate the effect of dietary composition on steroid dependent regulation of CYP activity we included a group of entire male pigs, fed dried chicory root.

Materials and Methods

Animals

Six entire male and four female pigs were raised under the same conditions, fed *ad libitum* with a common diet and slaughtered at an age of 164 days (approximately 130 kg). A second group of six entire male pigs was reared under similar conditions but feed an energy matched diet containing 10% dried chicory root 16 days prior to slaughter. All animals were crossbreeds between Landrace × Yorkshire sire and Duroc boars. Liver samples were taken at slaughter and stored at −80°C.

Preparation of liver microsomes

Frozen liver tissue was minced and homogenized in ice cold buffer containing 250 mM sucrose, 50 mM Tris–HCl, 150 mM KCl, 1 mM EDTA, 2 mM PMSF (pH 7.4) with a Polytron 2100 for 30 s. After 20 min centrifugation at 10 000 × ***g*** (4°C), the supernatant was centrifuged at 100 000 × ***g*** for 60 min at 4°C. The resulting pellet was suspended in TS-buffer (10 mM Tris-base, 0.9% NaCl, pH 7.4) for Western blotting, or resuspension buffer (50 mM Tris-base, 1 mM EDTA, 250 mM sucrose, 20% glycerol, pH 7.4) for activity measurements. Protein concentrations were determined using a BCA kit (Pierce, Thermo scientific, Rockford, IL, USA) according to the manufacturers' instruction.

Cytochrome P450-dependent activity

The activities of CYP1A, CYP1A2, CYP2A and CYP2E1 were measured as ethoxyresorufin *O*-deethylation (EROD), methoxyresorufin *O*-demethylation (MROD), coumarin hydroxylation (COH) and *p*-nitrophenol hydroxylation (PNPH), respectively.

The activity of CYP1A and CYP1A2 was measured according to Zamaratskaia and Zlabek (2009), but with modifications. Briefly 0.2 mg microsomal protein was incubated with either 2 μM ethoxyresorufin or methoxyresorufin in 50 mM phosphate buffer (pH 7.4) at 37°C. The reaction was started by adding 1 mM NADPH and stopped with 100% ice-cold methanol after 5 min for EROD or 10 min for MROD. The production of

resorufin was analysed by HPLC according to Zamaratskaia and Zlabek (2009).

The activity of CYP2A and CYP2E1 was measured according to Rasmussen et al. (2010), adapted to be used with microsomes. The activity of CYP2A was measured by incubating 0.4 mg microsomal protein with 0.2 mM coumarin in a buffer containing 50 mM Tris and 5 mM $MgCl_2$ (pH 7.4) at 37°C. The reaction was started by adding NADPH to a final concentration of 1 mM and was allowed to run for 30 min. The reaction was stopped by adding 20% trichloracetic acid in a ratio of 1:4, followed by 10 min centrifugation at 10 000 × ***g***. The supernatant was mixed with 0.1 M Tris–HCl (pH 9.0) and fluorescence was measured with an excitation wavelength at 390 nm and an emission wavelength at 450 nm.

For measuring activity of CYP2E1, 0.5 mg total microsomal protein was incubated with 0.2 mM *p*-nitrophenol in 100 mM potassium-phosphate buffer (pH 6.8) at 37°C. The reaction was started by adding NADPH to a final concentration of 1 mM. After 30 min the reaction was stopped by adding 20% trichloracetic acid in a ratio of 1:10. This was followed by 10 min centrifugation at 10 000 × ***g***, mixing of the supernatant 1:1 with 5 M NaOH and measuring of the absorbance at 515 nm.

All CYP activity measurements were done in duplicates. Enzyme activities were calculated by relating the amount of produced metabolite (resorufin for EROD and MROD, 7-hydroxycoumarin for COH and *p*-nitrocatechol for PNPH) to blank incubations with known metabolite concentrations.

Western blotting

Equal amounts of total protein were separated by SDS-polyacrylamide gel electrophoresis using a 12% Tris–Bis gel (Bio-Rad, Hercules, CA, USA) and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad). After blocking with 2% Tween20 in TBS-buffer (50 mM Tris-base, 0.5 M NaCl, pH 7.4) for 5 min, the membrane was incubated with primary antibodies diluted in TBS-buffer containing 0.1% Tween20 overnight at 4°C. After washing 2 × 10 min in TBS-buffer, the membrane was incubated with species specific Alexaflour448 attached secondary antibodies (DAKO, Glostrup, Denmark). Before visualizing on a Molecular Imager® FX (Bio-Rad) scanner the membrane was washed 6 × 5 min in TBS-buffer with 0.1% Tween20. The relative protein concentration was quantified with Quantity One (version. 4.5.2. Bio-Rad).

The investigated CYP isoforms were detected with commercially available polyclonal antibodies raised against a human epitope [The CYP1A2, 2A and 2E1 antibodies were purchased from Acris Antibodies (Herford, Germany), Santa Cruz (Santa Cruz, CA, USA) and Abcam (Cambridge, UK), respectively]. Due to the high homology between the aminoacid sequence of human and pig CYPs (Baranova et al. 2005; Messina et al. 2008; Skaanild and Friis 2005), the antibodies used should also recognize the respective porcine CYPs. This was confirmed as all antibodies identified one single band with a molecular weight corresponding to CYP.

Average protein expression from male pigs was arbitrarily set to 100 and the value of female pigs was expressed relative to the male pig group.

RNA isolation and reverse transcription

A total of 10 mg frozen liver tissue were homogenized in RLT-buffer for 2 × 20 s and mRNA isolated with a RNA isolation kit according to the manufacturer's instruction (Rnasey Mini Kit, WVR, Herlev, Denmark). The isolated RNA was dissolved in H_2O and converted to cDNA by reverse transcription using SuperScript II Rnase H Reverse Transcriptase and $Oligo(dT)_{12\text{-}18}$ Primer (Invitrogen, Carlsbad, CA, USA), according to the manufactures instruction. cDNA were diluted in RNAase free water and stored at −20°C until further use.

Quantitative PCR

Equal volumes of cDNA were used as template for polymerase chain reaction (PCR) using TaqMan probes. cDNA were mixed with TaqMan®2X Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) and specific primers and TaqMan probes for the selected gene. The PCR consisted of the following temperature profile: 50°C for 2 min, 95°C for 10 min followed by 40 cycles consisting of 95°C for 15 s and 60°C for 1 min.

Primers and TaqMan probes were designed with Primer Express (Version 2, Applied Biosystems) using pig specific sequences of genomic DNA. Probes were designed to span a splice site. A Blast search using NCBI's Basic Local Alignment Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) verified that the amplified sequence only obtained homology with the target gene.

Table 1. Sequences of primers and TaqMan probes used for PCR

Gene	Name	Sequence (5′ to 3′)	Reference
CYP1A2	Forward primer	CTGCAATTCCTGAGGAAAATGG	NM_001159614
	Reverse primer	CGCTTGTGATGTCCTGGATACA	
	Probe	AGGAGCGCTATCGGGACTTTGACAAGAA	
CYP2A19	Forward primer	TGGATGAGAACGGGCAGTTT	NM_214417
	Reverse primer	AGAGCTCCATTCTAGCCAGACCTT	
	Probe	TCTCCATCGGAAAGCGGTACTGTTTCG	
CYP2E1	Forward primer	CGGAAAGTTCAAGTACAGTGATCATT	NM_214421
	Reverse primer	GGCCCTCTCCGACACACA	
	Probe	CAAGGCATTTTCCGCAGGAAAGCG	
GAPDH	Forward primer	GTCGGAGTGAACGGATTTGG	Young et al. 2007
	Reverse primer	CAATGTCCACTTTGCCAGAGTTAA	
	Probe	CGCCTGGTCACCAGGGCTGCT	

Primers and probes are given in Table 1. Control samples with no mRNA and genomic DNA were analysed to confirm that no unspecific amplification occurred. All PCR's were done in duplicates.

The relative mRNA expression was calculated by relating the obtained values for threshold cycles to a standard curve obtained by running a serial dilution of one cDNA sample. The mRNA expression was normalized against the mRNA expression of glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and expressed as arbitrary units. The expression of GAPDH was not significantly different between genders. The average of the male group was arbitrarily set to 1 and the female pigs expressed relative to the male pig group.

Inhibition of substrate metabolism by steroids

Three pools of male and three pools of female microsomes were used to study steroid dependent inhibition of EROD, MROD and PNPH. Furthermore, three pools of microsomes from entire male pig feed dried chicory root were included. All microsome pools consisted of microsomes from two individual pigs.

Inhibition of substrate metabolism by 2 nM 17β-oestradiol, 2 nM oestrone, 55 nM androstenone or 55 nM 3β-OH androstenol was evaluated as previously described (Rasmussen et al. 2010). The individual steroids were dissolved in methanol giving a final methanol concentration of 0.1% when added to the reactions. Control incubations were run in parallel with 0.1% methanol. The pre-incubation step has previously been shown to be important in the studies of steroid dependent inhibition (Rasmussen et al. 2010). Thus, in the present study microsomal pools were first pre-incubated with steroid, in the above mentioned concentrations, and NADPH for 15 min at 37°C. Following pre-incubation, appropriate probe substrate together with additional NADPH (1 mM) were added and the reactions for EROD and MROD proceeded as described above (see section *Cytochrome P450-dependent activity*), and for PNPH as described in Rasmussen et al. (2010).

The effect of steroids on substrate metabolism was expressed as % of remaining activity relative to paired control samples incubated with 0.1% methanol.

Statistics

Differences in CYP activity, protein and mRNA expression were evaluated by Student's unpaired *t*-test.

One way ANOVA were used to test whether steroid treatment regulated enzyme activity. Tukey's *post-hoc* test was used to identify differences between steroid treatments within groups.

All statistic analyses were performed using the statistical software 'R' (version 2.11.0).

Results

Gender-related differences in cytochrome P450-dependent activity

Activities of CYP1A, 1A2, 2A and 2E1 in the microsomes are shown in Table 2. Microsomes from female pigs had approximately twofold greater EROD ($p < 0.01$) and MROD ($p < 0.01$) activity compared to the microsomes from entire male pigs. Furthermore COH activity was eightfold greater ($p < 0.05$) in the microsomes from female pigs than the microsomes from male pigs. PNPH activity was twofold greater in the microsomes from female pigs compared to the microsomes from male pigs. Yet, this difference was not statistically significant ($p = 0.13$).

Table 2. Cytochrome P450-dependent activity in the microsomes from entire male (n = 6) and female (n = 4) pigs. Activities are given as pmol/min/mg total protein

	Male	Female
CYP-dependent activity		
EROD	38.1 ± 4.6	62.4 ± 6.0**
MROD	8.2 ± 0.6	13.7 ± 1.7**
COH	3.0 ± 1.1	22.7 ± 7.4*
PNPH	20.6 ± 9.1	41.0 ± 5.5

Values are mean ± SEM. EROD, ethoxyresorufin *O*-deethylasese; MROD, methoxyresorufin *O*-demethylase; COH, coumarin hydroxylation; PNPH, *p*-nitrophenol hydroxylase. Level of significance: ** $p < 0.01$ and * $p < 0.05$.

Gender-related differences in mRNA and protein expression

To assess gender-related differences in mRNA and protein expression we performed RT-PCR and Western blotting.

The expression of CYP1A2 and CYP2A mRNA were significantly ($p < 0.01$ and $p < 0.05$, respectively) greater in female pigs that in male pigs (4.85 ± 0.56 and 6.65 ± 2.19 fold, respectively) (Fig. 1). There was no significant difference in the expression of CYP2E1 mRNA between male and female pigs.

Relative protein expression of CYP1A2, 2A and 2E1 in male vs. female pigs were 100.0 ± 12.3 vs 112.7 ± 4.7;

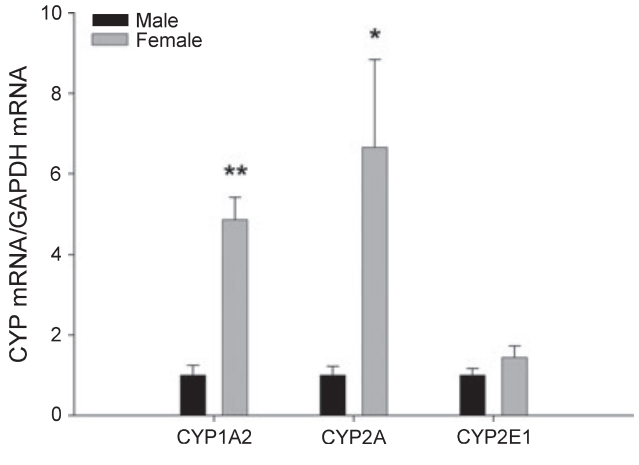

Fig. 1. Expression of mRNA of cytochrome P450 isoforms in entire male (n = 6) and female pigs (n = 4). The mRNA expression of the cytochrome P450s are normalized against the mRNA expression of GAPDH. Values are mean ± SEM expressed relative to entire male pigs with an average arbitrary set to 1. CYP, cytochrome P450; GAPDH, glyceraldehyde 3 phosphate dehydrogenase. Levels of significance: ** $p < 0.01$; * $p < 0.05$

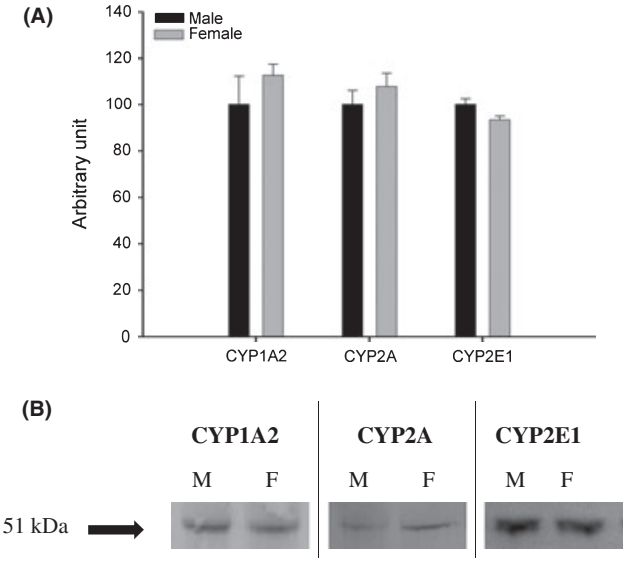

Fig. 2. (A) Protein expression of cytochrome P450 isoforms in microsomes from entire male (n = 6) and female (n = 4) pigs. Values are mean ± SEM expressed relative to entire male pigs with an average arbitrary set to 100. (B) representative blots probed with specific antibodies. M, male; F, female

100.0 ± 6.2 vs 107.9 ± 5.7 and 100.0 ± 2.6 vs 93.3 ± 1.6, respectively (Fig. 2). There were no significant differences, apart from a tendency ($p = 0.09$) in CYP2E1 expression.

Inhibition of CYP-dependent activity by steroids

There were no differences in EROD activity between control and steroid-treated microsomes from entire male and female pigs (Table 3). Yet all investigated steroids inhibited EROD activity in microsomes from chicory fed entire male pigs ($p < 0.01$). There was no effect on MROD activity when treating the microsomes with steroids. All investigated steroids inhibited PNPH in microsomes from entire male pigs ($p < 0.01$), whilst there was no inhibition in microsomes from female and entire male pigs fed chicory root.

Discussion

The present study showed that there were differences in cytochrome P450-dependent metabolism between entire male and female pigs. Female pigs exhibited significantly greater EROD, MROD and COH activity, and numerically greater PNPH activity. These observations are consistent with previous results showing that female pigs have greater CYP dependent activity (Skaanild and Friis 1999; Kojima et al. 2008). Yet the study by Skaanild and Friis (1999) investigated younger pigs (3.5 months), whilst the study by Kojima et al. (2008) investigated pigs not normally used for consumption (Meishan pigs). Zamaratskaia et al. (2006) investigated pigs determined for meat production. Female pigs at a live weight of 90 kg had greater CYP2A, but not CYP2E1 activity compared to those in male pigs. At 115 kg there were no differences in CYP2A activity, whilst CYP2E1 activity was greater in female pigs. In the present study, female pigs at approximately 130 kg showed greater CYP1A and 2A dependent activity than male pigs. Furthermore female pigs showed greater numeric CYP2E1 dependent activity. This shows that in sexually mature female pigs the activity of skatole metabolizing CYP is greater than in entire male pigs at the same age/live weight. This observation offers an explanation of lower skatole concentration in female pigs compared to entire male pigs at slaughter.

Less is known about the underlying regulation of this gender-related difference in skatole-metabolizing enzyme activities. Our results showed that the difference in activity was not linked to a difference in protein expression, as shown by Western blotting. Yet, Skaanild and Friis (1999) found a corresponding greater level of CYP dependent activity and protein expression in female pigs, compared to male pigs. Similarly, Kojima et al. (2008) found greater activity of CYP1A1 and CYP1A2 in sexual mature female Meishan pigs, compared to male pigs, which corresponded to greater

		Male	Female	Male (DCR)
EROD	Conc. (nM)			
17β-oestradiol	2	99.0 ± 1.8	104.2 ± 3.5	86.3 ± 2.5**
Oestrone	2	101.3 ± 3.0	99.3 ± 3.1	87.7 ± 3.0*
Androstenone	55	103.1 ± 1.5	100.7 ± 2.3	86.3 ± 1.3**
3β-OH androstenol	55	100.8 ± 3.2	102.0 ± 6.2	83.5 ± 1.5**
MROD				
17β-oestradiol	2	97.6 ± 3.4	100.3 ± 0.2	101.8 ± 1.7
Oestrone	2	101.9 ± 6.0	101.5 ± 2.2	102.9 ± 4.2
Androstenone	55	101.0 ± 4.3	98.2 ± 2.1	100.7 ± 4.2
3β-OH androstenol	55	94.0 ± 5.3	98.3 ± 6.1	102.9 ± 6.4
PNPH				
17β-oestradiol	2	86.4 ± 2.8**	99.9 ± 2.9	100.9 ± 1.6
Oestrone	2	87.3 ± 3.7*	101.2 ± 3.8	100.6 ± 2.0
Androstenone	55	84.7 ± 2.6**	104.7 ± 3.8	100.2 ± 3.8
3β-OH androstenol	55	73.8 ± 1.8**	102.0 ± 1.7	100.4 ± 4.4

Data are mean ± SEM percentage of control enzyme activity. EROD, ethoxyresorufin *O*-deethylasese; MROD, methoxyresorufin *O*-demethylase; PNPH, *p*-nitrophenol hydroxylase. Level of significance ** $p < 0.01$; * $p < 0.05$ different from control.

Table 3. Effect of testicular steroids on cytochrome P450-dependent activity determined using pools of microsomes from female and entire male pig, fed a common or chicory (DCR) added diet. Each pool consisted of microsomes from two individual animals. Values are given as % remaining activity compared to control samples without steroids. CYP dependent metabolism of PNPH, EROD and MROD were determined in the presence of methanol (control) or varying steroids in concentrations as indicated in the table. Before adding the substrate, microsomes were incubated with methanol or steroids for 15 min. Enzyme activities in the presence of methanol were regarded as 100%

protein and mRNA expression. This gender-related difference in protein and mRNA expression was only found in sexual mature pigs, and Kojima et al. (2008) concluded that the observation was due to down-regulation in male pigs. Thus, it is somewhat surprizing that the present observed gender-related differences in CYP activity did not resulted in observation of difference in protein expression, with the use of Western blotting. Furthermore, this observation seems even more surprizing given the large differences in CYP1A2 and 2A mRNA expression. Taken together these results suggest that the amounts of protein are not strictly regulated by the expression of mRNA and that enzyme activity is not strictly depending on protein expression. A study on human CYP showed that the activity of CYP2A6 and 2E1 is not correlated to mRNA expression, whilst EROD and MROD activity correlated strongly to the expression of CYP1A1 and 1A2 mRNA (Rodriguez-Antona et al. 2001). Yet, Skaanild and Friis (1999) found strong correlation between EROD activity and CYP1A2 protein expression, as well as between COH activity and CYP2A6 protein and mRNA expression. The authors found only very weak correlation between CYP2E1 dependent activity and protein expression. Further investigations are needed to elucidate the underlying mechanisms for the gender-related differences in CYP dependent activity.

Our results indicate that lower skatole levels in female pigs are due to greater skatole metabolism in the liver. To evaluate the direct regulation of skatole-metabolizing enzymes by gonadal steroids, *in vitro* incubations of the microsomes were used. In this study, we included four steroids. 17β-oestradiol and oestrone were chosen because male pigs are known to produce high levels of oestrogens compared to males of other species (Claus and Hoffmann 1980), and oestrogens are likely involved in the regulation of skatole metabolism (Zamaratskaia et al. 2007). Pigs at younger age (before puberty) with low levels of oestrogens and other gonadal steroids usually do not express high skatole levels. The levels of androstenone and its major metabolite 3β-OH androstenol might also be important in the determination of the rate of skatole metabolism. Androstenone was shown to block skatole-induced CYP2E1 expression (Doran et al. 2002). To our knowledge the effect of 3β-OH androstenol on CYP dependent activity has not previously been investigated. The present observation of inhibition of CYP2E1 dependent metabolism by 17β-oestradiol, oestrone, androstenone and 3β-OH androstenol in entire male pigs, but not in female pigs, suggest that the mechanism of CYP regulation by gonadal steroids is gender-dependent. This could also to some extent explain the greater skatole concentration normally found in entire male pigs. It has previously been suggested that the *in vitro* inhibitory effect of androstenone on CYP2E1 was due to some of its metabolites, particularly 3β-OH androstenol (Rasmussen et al. 2010). In support of this hypothesis our result show that 3β-OH androstenol inhibits CYP2E1 dependent activity to a greater extent than androstenone ($p = 0.08$). Yet, we cannot exclude that other mechanisms are responsible for this difference in the extent of inhibition. For example, 3β-OH androstenol can simply be a more potent inhibitor of CYP2E1 activity compared to its precursor androstenone.

The investigated steroids showed no inhibitory effect on EROD and MROD in entire male and female pigs. EROD and MROD have previously been used as specific substrates for porcine CYP1A1 and CYP1A2, respectively (Ptak et al. 2006). Yet a study characterizing EROD and MROD in porcine liver microsomes showed that the substrates may not be as specific as previously believed (Zamaratskaia and Zlabek 2009). MROD seems to be more dependent on CYP1A2 activity than EROD (Messina et al. 2008), whilst EROD seems to be more specific towards CYP1A1 activity (Chirulli et al. 2007).

In the present study we also investigated the effect of dietary compositions on steroid dependent inhibition of the CYPs. When comparing the inhibitory effect of 17β-oestradiol, oestrone, androstenone and 3β-OH androstenol in entire male pigs fed a common diet and entire male pigs fed an energy matched diet containing dried chicory root our results show different catalytically responses. CYP2E1 dependent activity was decreased in the presence of steroids in the microsomes from common fed pigs, whilst there was no change in activity in the microsomes from pigs feed chicory root. Furthermore, EROD activity was decreased by the investigated steroids in chicory fed pigs, whilst no changes were observed in common fed pigs. This shows that dietary composition changes the properties of the CYP. Yet, what causes this difference is unknown. We have previously suggested that a male and female version of the CYP exist, which is supported by the differences in inhibition found in the present study and other studies (Zamaratskaia et al. 2007; Rasmussen et al. 2010). Thus, it could be speculated that chicory root induce a shift towards expression of female CYP versions in entire male pigs with respect to CYP2E1. In rats the expression of two gender-specific CYP2C are shown to be regulated by the sexually dimorphic pattern of growth hormone secretion by the pituitary gland (reviewed by Waxman and O'Connor 2006). The existence of gender-specific CYP in pigs and regulation of their expression needs to be addressed in future investigations.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

The work presented here was performed in collaboration between all authors. Martin Krøyer Rasmussen was designing the experiment, conducted the sample preparation, RT-PCR, Western blotting, statistically analysed the data, interpreted the results and prepared the manuscript. Measurements of enzymatic activities were performed in collaboration between Martin Krøyer Rasmussen and Galia

Zamaratskaia. Galia Zamaratskaia contributed to the interpretation of data, discussion of the results and editing of the manuscript. Bo Ekstrand provided scientific leadership, contributed to designing the experiment and interpretation of data, discussion of the results and editing of the manuscript. All authors have read and approved the final manuscript.

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Paper III

In vivo effect of dried chicory root (*Cichorium intybus L.*) on xenobiotica metabolizing cytochrome P450 enzymes in porcine liver

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In vivo effect of dried chicory root (*Cichorium intybus* L.) on xenobiotica metabolising cytochrome P450 enzymes in porcine liver

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ABSTRACT

Cytochrome P450 (CYP) enzymes are widely studied for their involvement in metabolism of drugs and endogenous compounds. In porcine liver, CYP1A2, 2A and 2E1 are important for the metabolism of skatole. Feeding chicory roots to pigs is known to decrease the skatole concentration in plasma and fat. In the present study we investigated the effect of chicory on CYP mRNA and protein expression, as well as their activity. Male pigs were feed dried chicory root for 16 days before liver samples were collected. By the use of RT-PCR and Western blotting we showed that the mRNA and protein expression of CYP1A2 and 2A were increased in chicory fed pigs. The mRNA expression of CYP2E1 was increased, while there was no effect on protein expression. Activity of CYP1A2 and 2A were increased in chicory feed pigs; this was not the case for CYP2E1 activity. In conclusion; oral administration of chicory root for 16 days to pigs increased the mRNA expression of CYP1A2, 2A and 2E1; and the protein expression of CYP1A2 and 2A. The activities of CYP1A2 and 2A were increased.

1. Introduction

The cytochrome P450 enzymes (CYP) are a superfamily of enzymes important for the metabolism of endogenous compounds and xenobiotics. High concentrations of CYPs are mostly found in the liver, but also in the small and large intestine, lung and brain. CYPs are extensively studied for their involvement in drug metabolism and activation of pro-carcinogens. Inter-individual variation in e.g. CYP dependent drug metabolism has been observed (Zanger et al., 2008) and is often explained by the genetic profile of the individual. However there is growing evidence for a regulatory effect obtained by dietary compounds. The expression and activity of hepatic CYP can be up- or down-regulated by bioactive compounds from plants (reviewed by Chang, 2009). One extensively studied case is the inhibitory effect of grape fruit juice on CYP3A4 activity and consequently lower clearance of drugs metabolised by that CYP isoform (Kiani and Imam, 2007). An *in vitro* experiment using human hepatocytes showed that CYP2C19 activity was up-regulated upon treatment with extracts of St. Johns Wort, Common Valerian, *Ginkgo biloba* and Common Sage, while there was no effect of Cone Flower or Horse Chestnut (Hellum et al., 2009). The same study reported that CYP2E1 activity was up-regulated by St. Johns Wort, while all the previously mentioned plants had no or down-regulating effect on CYP2E1 activity.

The perennial herb chicory (*Cichorium intybus* L.) has been shown to posses several health beneficial properties (reviewed by Bais and Ravishankar, 2001) including anti-carcinogenic (Pool-Zobel et al., 2002), anti-inflammatory (Cavin et al., 2005), antioxidant (Gazzani et al., 2000), as well as protection against immunotoxicity induced by ethanol (Kim et al., 2002) and a prebiotic effect depending on its content of inulin (reviewed by Kolida et al., 2002). Chicory is used as a coffee substitute and the roots are often used as a source for inulin. Moreover, one group of documented bioactive component in chicory is sesquiterpene lactones (Rodriguez et al., 1976; Beek et al., 1990).

The aim of the present study was to investigate whether oral administrations of chicory root increases the activity and expression of hepatic CYP enzymes in entire (uncastrated) male pigs. Differences in CYP1A2, 2A and E1 dependent activity, mRNA and protein expression were evaluated in liver samples from the pigs. The selected enzymes are of particular importance in pigs because of their role in the metabolism of skatole, one of the major compounds responsible for boar taint (Zamaratskaia and Squires, 2009). Moreover, pigs could be used as a suitable model for humans, because their physiological characteristics are similar to humans (Lunney, 2007).

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Table 1
Composition and nutrient content of the control (CON) and experimental diet (DCR).

	CON	DCR
Components (% of total weight)		
Dried chicory root[a]	–	10.0
Barley	60.0	50.0
Soybean cake	16.7	19.0
Wheat	12.1	11.3
Oat	5.0	3.0
Vegetable fat	1.9	1.2
Melasses	1.0	3.2
Calculated analysis (g/kg)		
FEsvin[b]	1.0	1.0
Dry matter	863.6	871.3
Crude protein	156.0	161.8
Crude fat	42.2	32.0
Total fibre	165.1	226.1
Inulin	–	47.1
Tryptophan	2.0	2.1

[a] Dried at 65 °C.
[b] Feeding unit pig.

2. Materials and methods

2.1. Chemicals

Coumarin, *p*-nitrophenol, ethoxyresorufin, and methoxyresorufin were purchased from Sigma–Aldrich (St. Louis, MO, USA). The CYP1A2, 2A and 2E1 antibodies were purchased from Acris Antibodies (Herford, Germany), Santa Cruz (Santa Cruz, CA, USA) and Abcam (Cambridge, UK), respectively. Primers and probes were custom made by DNA Technology (Aarhus, Denmark). All chemicals and solvents were of analytical grade and were obtained from common commercial sources.

2.2. Experimental animals, feeding strategies and sampling

All animals were entire (uncastrated) male pigs of a crossbreed between Landrace × Yorkshire sire and Duroc boars. 20 pigs were raised under the same conditions, fed *ad libitum* with a commercially available diet (CON; Table 1) and kept in the same pen until 16 days before slaughter. 6 pigs were randomly allocated to the control group and kept in a separate pen where they remain fed *ad libitum* with the control diet (CON). The remaining 14 pigs were fed *ad libitum* with an energy matched diet containing 10% dried chicory root (DCR; Table 1) for the last 16 days before slaughter. At an age of 164 days (weighing approximately 130 kg) all pigs were slaughtered in a commercial slaughter house. After stunning with CO_2, the pigs were burned, scalded at approximately 60 °C and eviscerated and samples of the liver tissue were collected. All samples were taken approximately 20 min after stunning. The liver samples were stored at −80 °C until further analysis. There were no differences between the groups with regard to use of medicine, signs of diseases, health problems, carcass weight and lean meat content.

2.3. Preparation of liver microsomes

Liver microsomes were prepared by ultracentrifugation according to Rasmussen et al. (2010b,c). Briefly, tissue was homogenized in a buffer containing 250 mM sucrose, 50 mM Tris–HCl, 150 mM KCl, 1 mM EDTA, 2 mM PMSF (pH 7.4) and centrifuged at 10000 × *g* (4 °C). The supernatant was then centrifuged at 100000 × *g* for 60 min at 4 °C and the resulting pellet was suspended in appropriate buffer (100 mM Tris-base, 1% SDS, pH 9.5 for Western blotting, or 50 mM Tris-base, 1 mM EDTA, 250 mM sucrose, 20% glycerol, pH 7.4 for activity measurements). Total protein concentration was determined using a BCA kit (Pierce) according to the manufacturer's instruction.

2.4. Measurement of specific CYP activity

The activity of CYP1A was measured as the rates of *O*-dealkylation of ethoxyresorufin (EROD) or methoxyresorufin (MROD) to resorufin with a modified method according to Zamaratskaia and Zlabek (2009). 0.2 mg total protein was incubated in 0.5 ml 50 mM phosphate buffer (pH 7.4) at 37 °C with either 2 μM ethoxyresorufin or methoxyresorufin dissolved in dimethylsulfoxide (DMSO), giving a final concentration of DMSO at 0.4%. The reaction was started by adding 1 mM NADPH and stopped with 100% ice-cold methanol after 5 min for EROD or 10 min for MROD. The formation of resorufin was analysed by HPLC (Zamaratskaia and Zlabek, 2009).

The activity of CYP2A was measured as the rate of hydroxylation of coumarin (COH) to 7-hydroxycoumarin, while the activity of CYP2E1 was measured as the rate of hydroxylation of *p*-nitrophenol (PNPH) to *p*-nitrochatecol. Both assays were done according to Rasmussen et al. (2010b).

All CYP activity measurements were done in duplicates.

Table 2
Cytochrome P450 dependent activity in liver microsomes from entire male pigs feed control (CON) or experimental (DCR) diet. Activities are given as pmol/min/mg protein. EROD, ethoxyresorufin *O*-deethylase; MROD, methoxyresorufin *O*-demethylase; COH, coumarin hydroxylation; PNPH, *p*-nitrophenol hydroxylase. Level of significance: $^{**}p < 0.01$ and $^{*}p < 0.05$.

	CON	DCR
CYP-dependent activity		
EROD	38.1 ± 4.6	39.1 ± 3.8
MROD	8.2 ± 0.6	12.2 ± 0.8**
COH	3.0 ± 1.1	13.0 ± 1.7*
PNPH	20.6 ± 9.1	29.5 ± 3.9

The activity of the CON group has been published before (Rasmussen et al., 2010b).

2.5. Western blotting

Equal amounts of protein were separated on a 12% Bis-Tris gel (BioRad, Hercules, CA, USA) and electroblotted onto a PVDP membrane. After 5 min of blocking in TBS-buffer (50 mM Tris-base, 0.5 M NaCl, pH 7.4) with 2% Tween20, the membrane was treated with primary antibody diluted in TBS-buffer containing 0.1% Tween20 overnight at 4 °C. The membrane was then washed 3× 10 min in TBS-buffer containing 0.1% Tween20 and incubated for 90 min at room temperature with a secondary antibody attached with Alexafluor488. Before scanning on a Molecular Imager® FX (Bio-Rad, Hercules, CA, USA) scanner, the membrane was washed 6× 5 min in TBS-buffer with 0.1% Tween20. The primary antibodies used are raised against a human epitope and have previously been shown to be specific in identifying porcine CYPs (Rasmussen et al., 2010b).

All samples to be compared were quantified relative to a standard sample. The average of the control group was arbitrary set to 100 and the experimentally fed group expressed relative to that.

2.6. Reverse transcription

RNA was isolated with Rnasey Mini Kit (WVR, Herlev, Denmark) according to the manufactures instruction. The isolated RNA was quantified by measuring the absorbance at 260 nm and purity assessed by the 260 nm/280 nm ratio, which was always above 1.9. Reverse transcription using SuperScript II Rnase H Reverse Transcriptase and Oligo(dT)$_{12-18}$ Primer (Invitrogen, Carlsbad, CA, USA) were used to convert RNA to cDNA. cDNA were diluted in RNAase free water and stored at −20 °C until further use.

2.7. Quantitative PCR

The amount of cDNA was quantified by polymerase chain reaction (PCR) using TaqMan probes. cDNA, primers and probes were mixed with TaqMan®2X Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The sequence of primers and probes were the same as used by Rasmussen et al. (2010b). The PCR consisted of the following temperature profile: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

The relative mRNA expression was calculated from the obtained values for threshold cycles related to a standard curve obtained by running a serial dilution of one cDNA sample. The relative mRNA amount was normalised against the mRNA expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and expressed as arbitrary units. The mRNA expression of GAPDH was not affected by the feed. Average of the control group was arbitrary set to 1 and the experimentally fed group expressed relative to that.

For all PCR analysis, samples with no DNA and genomic DNA were analysed to confirm that no unspecific amplification occurred. All PCRs were done in duplicates.

2.8. Statistical analysis

Student's unpaired *t*-test was used to test for differences between groups.

3. Results

3.1. Cytochrome activity in microsomes

In pigs fed with chicory root the CYP2A dependent COH activity was increased approximately 4 times ($p < 0.01$), while the CYP2E1 dependent PNPH activity did not differ ($p > 0.05$) between the two groups (Table 2). Two different substrates were used to evaluate the activity of CYP1A. Ethoxyresorufin has been shown to be metabolised by CYP1A1, while methoxyresorufin is more specific to CYP1A2 metabolism (Messina et al., 2008; Chirulli et al., 2007). There was no difference ($p > 0.05$) in EROD activity in the micro-

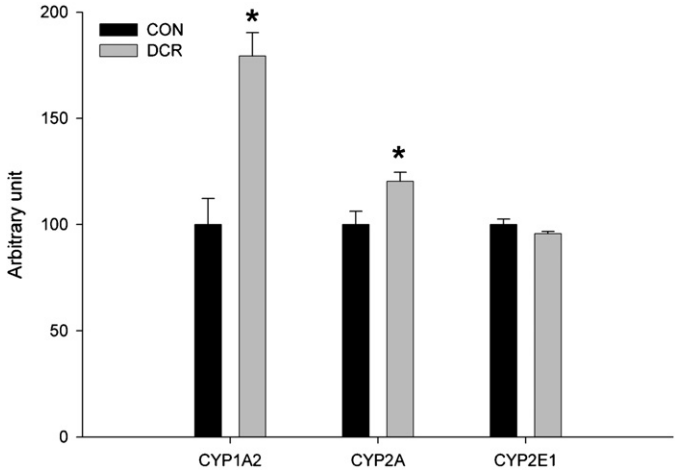

Fig. 1. Protein expression of cytochrome P450 isoform 1A2, 2A and 2E1 in microsomes from entire male pigs feed control diet (CON) or experimental diet (DCR). Levels of significance: $^{*}p < 0.05$.

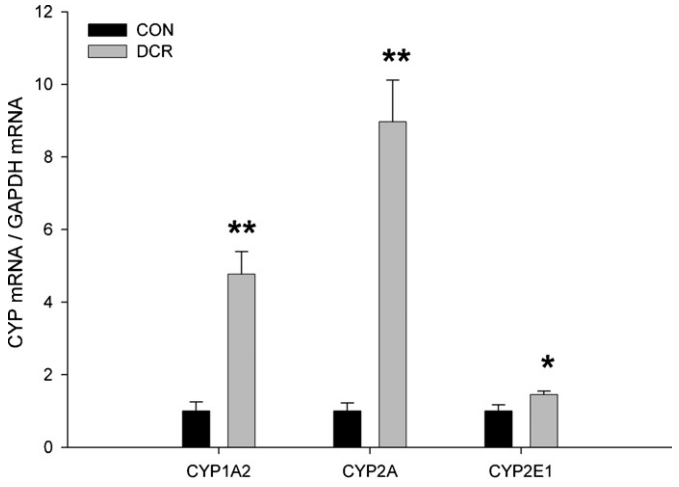

Fig. 2. mRNA expression of cytochrome P450 isoform 1A2, 2A and 2E1 in liver tissue from entire male pigs feed control diet (CON) or experimental diet (DCR). Levels of significance: $^{**}p < 0.01$; $^{*}p < 0.05$.

somes from entire male pigs fed either chicory root or control diet (Table 2). The MROD activity was increased approximately 1.5 times ($p < 0.01$) in the microsomes from entire male pig fed chicory root compared to control fed pigs (Table 2).

3.2. Protein and mRNA expression of CYP1A2, 2A and 2E1

The expression of CYP1A2 and CYP2A in entire male pigs fed chicory root was 79 and 20% greater, respectively ($p < 0.05$) (Fig. 1) compared to control pigs. There were no differences between the expression of CYP2E1 in the two groups (Fig. 1).

Semi quantitative PCR showed that the group of pigs fed chicory root had greater mRNA expression of CYP1A2 (4.7 ± 0.6 fold; $p < 0.01$), 2A (9.0 ± 1.2 fold; $p < 0.01$) and 2E1 (1.5 ± 0.1 fold; $p < 0.05$) (Fig. 2).

4. Discussion

The regulation of CYP activity involves multiple mechanisms at different levels and is mainly studied in humans, rats and mice. However, the information about regulation of CYP by bioactive compounds in pigs is still limited. This is to the best of our knowledge the first study to investigate the effect of dietary compounds on the expression and activity of hepatic CYP in pigs. We showed that oral administration of dried chicory root for 16 days increased the mRNA levels of CYP1A2, CYP2A and CYP2E1 and protein expression of CYP1A2 and CYP2A. Additionally, the MROD and COH were greater in the pigs fed chicory root.

Porcine CYP2A and 2E1 have been extensively studied for their involvement in the skatole metabolism (reviewed by Zamaratskaia and Squires, 2009). Moreover CYP1A2 has also been shown to metabolise skatole in both humans and pigs (Matal et al., 2009). *In vitro* experiments have showed that in microsomes prepared from male pigs the activity of CYP2E1 was inhibited in the presence of testicular steroids (Rasmussen et al., 2010a,b; Zamaratskaia et al., 2007). Decreased skatole metabolism in mature male pigs leads to accumulation of high skatole concentrations in the adipose tissue, negatively affecting the sensory quality of the meat. Feeding chicory root to sexually mature male pigs has previously been shown to decrease the concentration of skatole in both plasma and adipose tissue (Hansen et al., 2002; Hansen et al., 2006; Byrne et al., 2008; Lanthier et al., 2006). This effect has partly been ascribed to a prebiotic effect due to high inulin content. However, our study for the first time suggests that chicory root may increase the hepatic skatole-metabolism through the induction of CYP expression and activity. Feeding entire male pigs with chicory root for 16 days increased the CYP1A2 and CYP2A dependent activity. Moreover, we showed that the protein and mRNA expression of CYP1A2 and CYP2A were also increased. In the present study we found no effect of chicory root on CYP2E1 dependent activity and protein expression. However, the expression of CYP2E1 mRNA was increased 1.5 fold. Taken together these results show, that transcription and translation of CYP2A and CYP1A2 are increased by chicory root, while CYP2E1 is not. Increased expression of CYP proteins are in most cases achieved by de novo synthesis regulated by the involvement of cytosolic receptors like pregnane X receptor (PXR), constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AhR) (reviewed by Honkakoski and Negishi, 2000).

It is worth noting that the specificities of the substrates used to asses CYP activity in this study for the different pig CYP450s are debated. Thus, it has been suggested that *p*-nitrophenol can be metabolized by both CYP2E1 and CYP2A in porcine liver microsomes (Skaanild and Friis, 2007). Furthermore the specificity of EROD and MROD in pigs is not fully elucidated (Zamaratskaia and Zlabek, 2009). Nevertheless, the used substrates are commonly used to asses specific CYP activity and we believe that the results provide solid evidence on the importance of chicory root in the modulation of porcine CYP450s.

The sesquiterpene lactone artemisinin, origin from the genus *Artemisia*, has showed to be an agonist for CAR and thereby regulating CYP expression (Simonsson et al., 2006). The experimental set-up used in the present study could not be used to elucidate the mechanisms underlying the observed CYP induction. We suggest that the induction could be due to the presence of one or more agonists for the PXR, CAR or AhR, given that chicory contains sesquiterpene lactones. However, several other possible mechanisms of CYP induction exists including indirect effect of altered hormonal or endogenous substrate concentrations through a prebiotic effects on the intestinal microbial flora, or altered mRNA turnover rates. However further studies are needed to elucidate the underlying mechanisms.

5. Conclusion

The current study provided the first evidence that feeding dried chicory root to pigs induced the mRNA expression of CYP1A2, CYP2A and CYP2E1. Protein concentrations of CYP1A2 and CYP2A as well as MROD and COH rates were also significantly increased.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Paper IV

Feeding dried chicory root to pigs decreases androstenone accumulation in fat by increasing hepatic 3β hydroxysteroid dehydrogenase expression

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Feeding dried chicory root to pigs decrease androstenone accumulation in fat by increasing hepatic 3β hydroxysteroid dehydrogenase expression

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ABSTRACT

The present study investigated the *in vivo* effect of chicory root on testicular steroid concentrations and androstenone metabolizing enzymes in entire male pigs. Furthermore, the effect on skatole and indole concentrations in plasma and adipose tissue was investigated. The pigs were divided into two groups; one receiving experimental feed containing 10% dried chicory root for 16 days before slaughter, the control group was fed a standard diet. Plasma, adipose and liver tissue samples were collected at slaughter. Plasma was analyzed for the concentration of testosterone, estradiol, insulin-like growth factor 1 (IGF-1), skatole and indole. Adipose tissue was analyzed for the concentration of androstenone, skatole and indole, while the liver tissue was analyzed for mRNA and protein expressions of 3β-hydroxysteroid dehydrogenase (3β-HSD), sulfotransferase 2A1 and heat-shock protein 70 (HSP70). The results showed that the androstenone concentrations in the adipose tissue of chicory fed pigs were significantly ($p < 0.05$) lower and indole concentrations were higher ($p < 0.05$) compared to control fed pigs. Moreover the chicory root fed pigs had increased mRNA and protein expression of 3β-HSD and decreased HSP70 expression ($p < 0.05$). Testosterone and IGF-1 concentrations in plasma as well as skatole concentrations in adipose tissue were not altered by dietary intake of chicory root. It is concluded that chicory root in the diet reduces the concentration of androstenone in adipose tissue *via* induction of 3β-HSD, and that these changes were not due to increased cellular stress.

1. Introduction

Steroids of testicular origin are known for their diverse effect in pigs. Male pigs possess high concentrations of androstenone (5α-androst-16-en-3-one), estradiol and testosterone in plasma [1]. This leads to increased levels of the main boar taint-related compounds androstenone and skatole (3-methylindole) in adipose tissue in sexually mature male pigs and decreased meat quality. Clearance of endogenous compounds and xenobiotics by the liver is usually conducted in two steps: Phase I and II [2]. Phase I typically consists of an oxidation of the compound usually catalyzed by cytochrome P450 (CYP450) enzymes, while Phase II is conducted by a more diverse group of enzymes and consists of conjugation with a hydrophilic group, like glucuronidation, sulfoconjugation or glucosidation. The outcome of Phase I and/or II metabolism is often the elimination of the compound by excretion. In pigs, the endogenous boar taint compounds androstenone and skatole are processed by Phase I and II enzymes before excretion *via* the urine [3]. Hepatic metabolism of androstenone in pigs is mainly mediated by the 3β-hydroxysteroid dehydrogenase (3β-HSD) followed by sulfoconjugation by hydroxysteroid sulfotransferase isoforms 2A1(SULT2A1) or 2B1 [3]. The Phase I metabolism of skatole is mainly mediated by hepatic CYP1A2, CYP2A and CYP2E1 [4,5].

There is a strong interaction between testicular steroids and skatole metabolism in the liver due to involvement of testicular steroids in the regulation of CYP450. *In vitro* experiments with porcine primary hepatocytes have shown that androstenone increases the protein expression of CYP2A6, while estradiol sulfate and testosterone had no effect on CYP2A6 protein expression [6]. Moreover, in porcine primary hepatocytes, androstenone was shown to inhibit a skatole induced increase in CYP2E1 expression [7]. However, androstenone alone did not affect CYP2E1 expression. Kojima et al. [8] showed that administration of testosterone propionate to pigs decreased the expression of CYP1A. The suggestion that CYP450 expression in pigs is affected by steroids is further supported by studies showing difference in CYP450 expression/activity with respect to gender [9–13], castration [14] and immunocastration (active immunization against GnRH) [11,15]. These studies provide strong evidence that androgens and estrogens have a down-regulating effect on CYP450 expression and activity.

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Apart from testicular steroids, dietary composition has been shown to modulate CYP450 expression in humans [16], rats [17] and pigs [18]. Oral administration of chicory root to male pigs was shown to increase the expression and activity of CYP1A2 and 2A. The observed increase in CYP450 expression in chicory fed pig and the suggestion that steroids down-regulate CYP450 expression, led to the hypothesis that chicory fed pigs has decreased concentrations of testicular steroids and increased expression of steroid metabolizing enzymes.

This study is an extension of previous work from our research group showing that administration of chicory root to sexually mature pigs increases CYP2A, 1A2 and 2E1 mRNA expression, as well as CYP2A and CYP1A2 activity and protein expression [18]. The aim of the present study was to investigate the effect of oral administration of chicory root on steroid concentration in plasma and adipose tissue and steroid metabolism in the liver. The mRNA and protein expression of 3β-HSD and SULT2A1 was analyzed. Increased expression of phase I and/or II enzymes are often related to toxicological responses, so to evaluate if this is the reason for the changes related to consumption of chicory we investigated, the mRNA and protein expression of heat-shock protein 70 (HSP70). The expression of HSP70 is related to increased hepatic cellular stress [19,20].

2. Materials and methods

2.1. Animals and sampling

Experimental design and slaughter procedure is described in Rasmussen et al. [18]. Briefly, 22 entire male pigs were randomly assessed to experimental (16 pigs) or control (6 pigs) feeding strategies 16 days prior to slaughter. The pigs receiving experimental feed were given a diet containing 10% dried chicory root, while the control group remained fed with the energy matched control diet. The two groups of pigs were kept in separate pens. All pigs were fed *ad libitum* and slaughtered at the same age in a commercial slaughter house. Blood samples were collected at the time of killing, while liver and adipose tissue samples were collected approximately 20 min later at the opening of the carcass. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Due to an error during the slaughter procedure, only 14 tissue samples from the experimentally fed pigs were obtained. Blood samples were kept on ice until arrival at laboratory. At arrival, plasma was separated from blood cells by centrifugation at 2000 × *g* for 15 min and stored at −20 °C.

2.2. Determination of testosterone, estradiol and IGF-1

Testosterone concentration in plasma was measured using a commercial RIA kit (TKTT, Diagnostic Products Corporation, Los Angeles, CA, USA), according to the manufacturer's instructions.

IGF-1 concentration in plasma was measured using a commercial EIA kit (DSL-10-2800, Diagnostic System Laboratories, Webster, TX, USA) in accordance with the manufacturer's instructions.

Estradiol concentration in plasma was measured using a commercial EIA human salivary kit (1-3702, Salimetrics, State College, PA, USA), through an adaptation of the manufacturer's protocol [21]. In brief, plasma samples were diluted with equal volume water and then kept overnight at 4 °C. 200 μl of the plasma mixtures were then added to the pre-coated wells and incubated 1 h at room temperature on a rotator (500 rpm) and, additionally, 1 h without rotation. After the pre-incubation, all following steps were performed in accordance with the manufacturer's instructions.

2.3. Determination of skatole, indole and androstenone

The determination of skatole and indole concentrations in plasma was performed by UHPLC, through an adaptation of our previous method [22]. Chromatography was carried out on a LaChrom Ultra system (Merck), consisting of a binary pumping system (L-2160U), autosampler (L-2200U), column oven (L-2300), fluorescence detector (L-2485U) and EZ Chrom Elite (Version 3.2.1) software. In brief, plasma samples were mixed with equal volume of acetone and mixed for 30 s. The samples were then kept at −20 °C for 20 min and centrifuged for 20 min at 12.000 × *g* (4 °C). The clear supernatants were injected on a Hibar Purospher STAR (50 mm × 2.1 mm, 2 μm) column (Merck). The mobile phase was delivered isocratically, using 45% methanol and 55% H_2O, both with 0.1% acetic acid. At a flow rate of 0.6 ml/min, indole and skatol were eluted after approximately 1.07 and 2.13 min, respectively. Fluorescence detection was performed with an excitation wavelength of 275 nm and an emission wavelength of 360 nm.

Concentrations were calculated using a standard curve of skatole and indole dissolved in H_2O and measured under the same conditions.

In order to analyze skatole, indole and androstenone in fat, the samples were first liquefied in a microwave oven at 300 W for 3 min. Then 150 μl liquid fat was mixed with 750 μl methanol (containing 0.33 μg/ml androstenone as internal standard) and incubated at 60 °C for 5 min before vortexing for 30 s. After 60 min incubation at −20 °C, the samples were centrifuged at 4500 × *g* for 5 min. For analysis of indole and skatole, the supernatant was analyzed by HPLC according to Chen et al. [23]. For analysis of androstenone, 140 μl of supernatant was mixed with 4.4 μl H_2O, 10 μl BF_3 and 30 μl 2% dansylhydrazine (w/v) and incubated for 5 min before performing HPLC analysis according to Chen et al. [23].

2.4. RNA isolation and semiquantitative PCR

RNA isolation, reverse transcription and PCR was done according to Rasmussen et al. [13]. Briefly, RNA was isolated from approximately 10 mg of homogenized liver tissue using a commercial RNA isolation kit (Rnasey Mini Kit, WVR, Herlev, Denmark), according to the manufacture's instruction. The concentration of isolated RNA was estimated by measuring the absorbance at 260 nm, and the purity assessed by the ration between the absorbance at 260 nm and 280 nm, this ratio was always above 1.8. Equal amount of the isolated RNA was converted to cDNA by the use of SuperScript II Rnase H Reverse Transcriptase and Oligo(dT)12–18 Primer (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. The relative mRNA content of the samples was assessed by the use of PCR with specific primers and a TaqMan probes. Equal volumes of cDNA were mixed with specific primers, TaqMan probes and TaqMan® 2× universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The PCR reaction was conducted in duplicates using a ABI 7900 HT sequence detection system (Applied Biosystems, Carlsbad, CA, USA) under the following conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Primers and TaqMan probes (Table 1) were designed using Primer Express (Version 2, Applied Biosystems, Carlsbad, CA, USA). To test for unspecific DNA amplification, samples with genomic DNA or H_2O were subject to the PCR. Relative mRNA expression was calculated by the obtained Ct values and normalized against the mRNA expression of GAPDH. The expression of the control group was arbitrarily set to 1.

Table 1
Primers and probes for PCR.

Gene	Name	Sequence (5′–3′)	Ref.
3β-HSD	Forward primer	GGGCGAGAGACCGTCATG	ENSSSCG00000006719
	Reverse primer	ACGCTGGCCTGGACACA	
	Probe	AGGTCAATGTGAAAGGTACCCAGCTC	
SULT2A1	Forward primer	TTCCAGGAGAAGATGGCAGATC	ENSSSCG00000003130
	Reverse primer	AGGATTGGGAAGTTTGTGAACATT	
	Probe	TCCTCAAGAGCTGTTCCCATGGCAA	
HSP70	Forward primer	GGCAAGGCCAACAAGATCAC	[46]
	Reverse primer	TTCTCAGCCTCCTGCACCAT	
	Probe	ACAAGGGCCGCCTGAGCAAGG	
GAPDH	Forward primer	GTCGGAGTGAACGGATTTGG	[47]
	Reverse primer	CAATGTCCACTTTGCCAGAGTTAA	
	Probe	CGCCTGGTCACCAGGGCTGCT	

2.5. Protein expression

Microsomes were prepared by homogenization of frozen liver samples in Tris–sucrose buffer (10 mM Tris–HCl, 250 mM sucrose, pH 7.4) and ultracentrifugation according to Rasmussen et al. [24]. Cytosolic proteins were detected in the liquid phase origin from the microsome preparation. Before the cytosolic fraction was subjected to Western blotting, trichloroacetic acid was added to a final concentration of 12% and left on ice for 10 min. The precipitated proteins were isolated by 20 min centrifugation at 20.000 × *g* (4 °C) and washed twice with ice cold acetone. The final pellet was redissolved in a buffer containing 100 mM Tris-base with 4% SDS (pH 9.5).

Total protein concentrations in the samples were detected using a BCA kit (Pierce) according to the manufacturer's instruction. Equal amounts of total proteins were subject to Western blotting, conducted according to Rasmussen et al. [24]. By using this approach the relative protein expression was normalized against the total protein concentration of the samples, to asses equal loading of total protein amounts, blotting efficiency and uniformity all blots were stained with Ponceau S and visual inspected before use. This strategy has previously been used to investigate relative changes in specific protein expression between groups [25,26].

For detection of 3β-HSD we used the R1484 antibody kindly donated by Professor J.I. Mason (Division of reproductive and developmental science, University of Edinburgh, Scotland). The SULT2A1 was detected with an antibody purchased from Santa Cruz (Santa Cruz, CA, USA), while HSP70 was detected with an antibody from Abcam (Cambridge, UK). Representative Western blots of all antibodies are given in Fig. 1.

The average protein expression of control feed pigs was arbitrary set to 100 and the value of experimental feed pigs expressed relative to this.

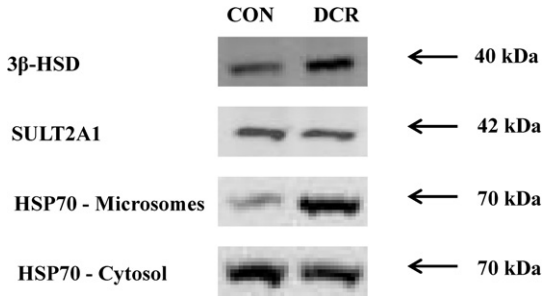

Fig. 1. Representative Western blots. CON: control feed pigs; DCR: chicory feed pigs. Arrows indicate position of molecular weight.

2.6. Statistical analysis

Differences between groups were assessed by Student's unpaired *t*-test. The differences were considered significant at $p < 0.05$.

3. Results

3.1. Concentrations of androstenone, testosterone, estradiol and IGF-1

Pigs fed chicory root had significantly lower concentrations of androstenone in adipose tissue samples ($p < 0.05$; Table 2). Furthermore, there was a tendency ($p < 0.1$) toward lower estradiol concentrations in plasma of chicory fed pig. Neither testosterone nor IGF-1 was altered by the presence of chicory root in the diet.

3.2. Hepatic expression of 3β-HSD, SULT2A1 and HSP70

The group of pigs fed chicory root showed increased 3β-HSD mRNA (+318%; $p < 0.01$; Fig. 2) and protein (+15%; $p < 0.05$; Fig. 3) expression compared to control feed pigs. There was no difference in mRNA and protein expression of SULT2A1 between chicory and control fed pigs ($p > 0.05$).

The chicory fed pigs had 38.6% lower mRNA expression of HSP70 compared to the control group ($p < 0.05$; Fig. 4A). Furthermore, protein expression of HSP70 in the chicory fed group was 25.0% ($p < 0.05$; Fig. 4B) and 16.3% ($p > 0.05$; Fig. 4C) lower in microsomes and the cytosolic fraction, respectively, compared to control.

3.3. Concentration of skatole and indole in plasma and fat

Pigs fed chicory root had increased concentrations of indole in adipose tissue ($p < 0.01$), while no differences were observed in plasma ($p > 0.05$; Table 3). There were no differences in skatole concentrations in plasma and adipose tissue between control and chicory fed pigs ($p > 0.05$; Table 3). Irrespectively of feed, all investigated animals showed concentrations of skatole well below

Table 2
Effect of oral administration of chicory root on concentrations of androstenone, testosterone, estradiol and IGF-1 in entire male pigs.

	CON	DCR	*p*-Value
Androstenone (ng/ml)	2.3 ± 0.7	0.9 ± 0.1	0.02
Testosterone (ng/ml)	7.8 ± 1.9	5.1 ± 1.0	0.19
Estradiol (pg/ml)	24.9 ± 10.7	10.3 ± 2.5	0.06
IGF-1 (ng/ml)	343.8 ± 63.9	394.4 ± 22.1	0.34

CON: control fed pigs ($n = 6$), DCR: dried chicory root fed pigs ($n = 16$). Values are mean ± standard error.

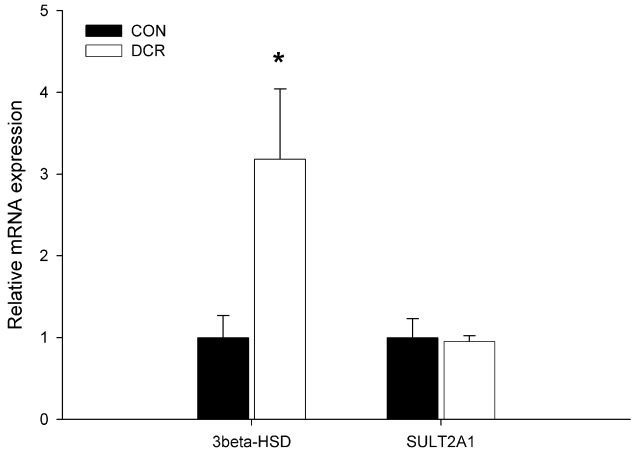

Fig. 2. Relative mRNA expressions of hepatic 3β-HSD and SULT2A1. CON: control fed pigs ($n = 6$), DCR: dried chicory root fed pigs ($n = 14$). Values are mean ± standard error. Levels of significance: $^{*}p < 0.05$.

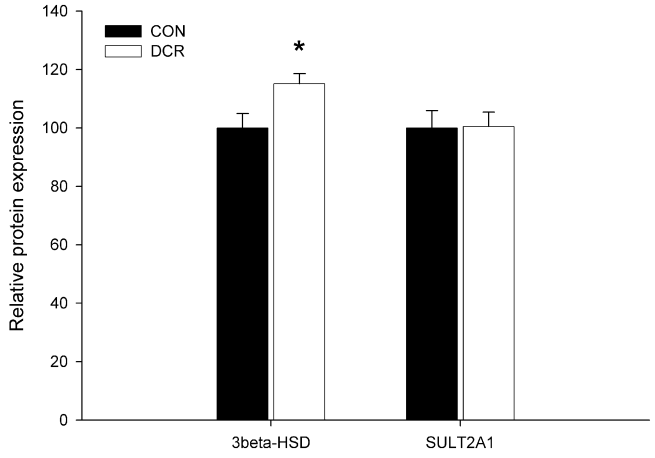

Fig. 3. Relative protein expression of hepatic 3β-HSD and SULT2A1. CON: control fed pigs ($n = 6$), DCR: dried chicory root fed pigs ($n = 14$). Values are mean ± standard error. Levels of significance: $^{*}p < 0.05$.

Table 3
Effect of oral administration of chicory root on concentrations of skatole and indole in adipose tissue and plasma of entire male pigs.

	CON	DCR	*p*-Value
Fat (ng/ml)			
Skatole	49.3 ± 19.6	44.2 ± 4.1	0.72
Indole	24.7 ± 7.8	59.3 ± 6.7	0.01
Plasma (ng/ml)			
Skatole	4.1 ± 1.0	5.5 ± 0.8	0.34
Indole	3.5 ± 0.5	5.3 ± 0.8	0.26

CON: control fed pigs ($n = 6$); DCR: dried chicory root fed pigs ($n = 16$). Values are mean ± standard error.

the threshold value used for boar taint detection (0.20–0.25 ppm; [27]).

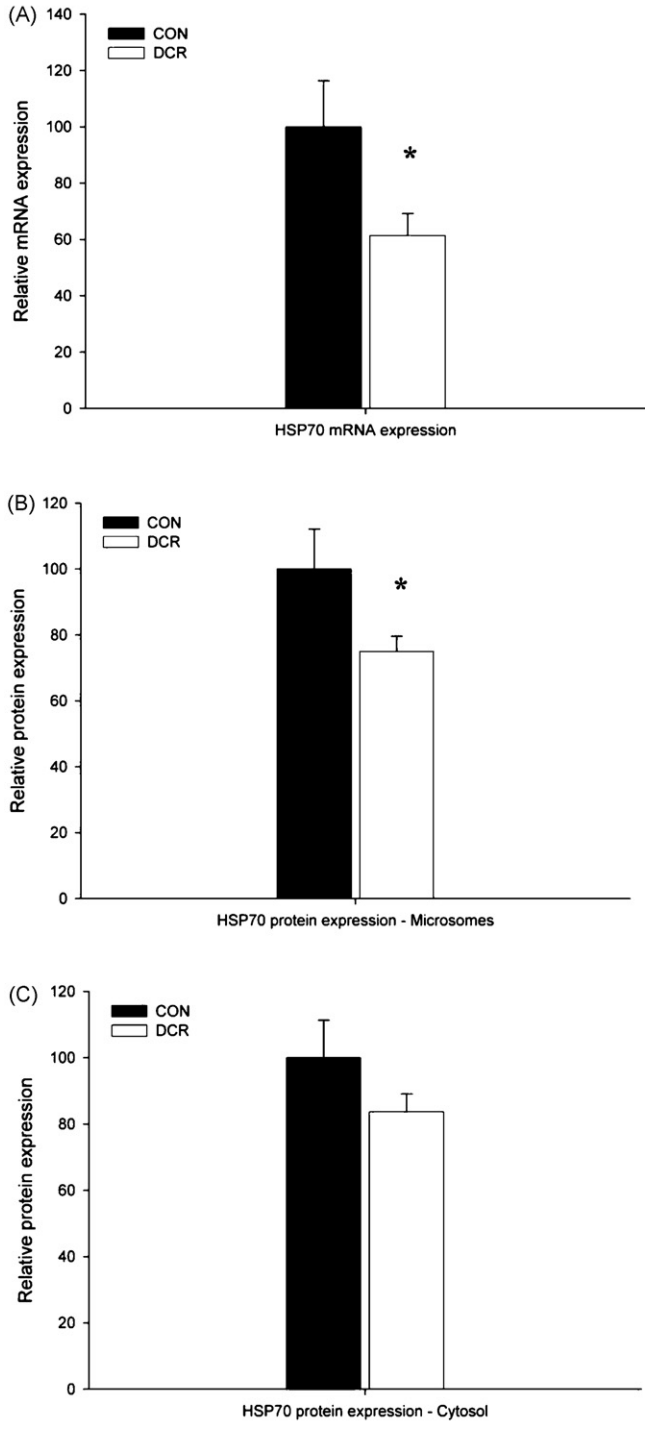

Fig. 4. Relative expression of hepatic heat-shock protein 70. (A) mRNA expression; (B) protein expression in microsomes and (C) protein expression in cytosolic phase. CON: control fed pigs ($n = 6$), DCR: dried chicory root fed pigs ($n = 14$). Values are mean ± standard error. Levels of significance; $^{*}p < 0.05$.

4. Discussion

The present study is an extension of our earlier work investigating the *in vivo* effects of feeding chicory root to entire male pigs. We have previously shown that pigs fed chicory root have increased mRNA expression of CYP1A2, 2A and 2E1 and that protein expression and enzyme activity of CYP1A2 and 2A are increased [18]. In the present study we showed that chicory fed pigs have decreased accumulation of androstenone in the adipose tissue, as well as increased levels of mRNA and protein expression of 3β-HSD.

High concentration of androstenone in pig adipose tissue is associated with decreased meat quality due to its unpleasant odor. Androstenone is produced in great amounts in the testes at the onset of puberty. The standard procedure to avoid increased androstenone accumulation in adipose tissue is to surgically castrate male piglets [14]. However, alternatives to surgical castration

are intensively studied due to increased concern about unfavorable effects of castration to animal health and welfare. These alternatives should be acceptable with respect to welfare, sustainable use of natural recourses and palatability of pork products. Different feeding regimes have been investigated for their ability to decrease androstenone accumulation in pigs. Dietary supplement of raw potato starch showed no effect on androstenone accumulation in adipose tissue [28,29]. In a study by Hansen et al. [30], lower androstenone concentration in blood was found in pigs fed crude chicory root for 9 weeks. The same study showed that addition of pure inulin to the feed had no effect on androstenone accumulation. Furthermore, addition of lupine to the feed decreases the androstenone concentration in fat [31]. However, other studies investigating androstenone accumulation have failed to show an effect of dietary composition [32].

It is generally accepted that variations in androstenone levels are due to the difference in the balance between the testicular synthesis and hepatic metabolism [3]. In the present study we found lower concentrations of androstenone in adipose tissue of pigs fed chicory root for 16 days. To elucidate the mechanism of this reduction, we measured mRNA and protein expression of two main androstenone-metabolizing enzymes in the liver. As we predicted in our hypothesis, the mRNA and protein expression of the Phase I androstenone metabolizing enzyme 3β-HSD were increased in the pigs fed chicory root. This is in accordance with earlier studies, showing that androstenone accumulation in fat is negatively correlated to the protein expression of 3β-HSD in the liver [33]. High 3β-HSD expression and activity enhance androstenone metabolism and thereby decrease the accumulation of androstenone in adipose tissue. Nicolau-Solano and Doran [34] showed that *in vitro* 3β-HSD protein expression in primary porcine hepatocytes increased upon treatment with androstenone and estrone sulfate. This effect was observed in hepatocytes isolated from heavier pigs (90 kg) and only in a limited range of androstenone and estrone sulfate concentrations. In the present *in vivo* study we showed that the pigs fed chicory root had lower androstenone concentrations and a tendency toward lowering of estradiol concentrations in plasma, suggesting that a similar mechanism is involved in the regulation of both testicular steroids.

Hepatic SULT2A1 is another important enzyme in the metabolism of androstenone. Expression and activity of SULT2A1 were shown to be related to the capacity of androstenone to accumulate in fat [35]. The present study demonstrated no effect of dietary supplement with chicory root on SULT2A1 expression. Thus, the difference in androstenone levels between chicory root and control fed pigs is not related to SULT2A1 expression.

Plasma levels of testosterone and IGF-1 did not differ between the two groups of pigs. This is an important finding. Usually, a reduction in androstenone levels is paralleled by a reduction in anabolic hormones including testosterone. This disadvantage was observed during selection experiments against high androstenone levels [36,37]. Active immunization against gonadotropin-releasing hormone, an alternative to surgical castration, does not selectively reduce the levels of androstenone, but blocks the production of all testicular steroids [38]. IGF-1 concentrations in plasma are also lower in immunized pigs compared to entire male pigs [39]. In contrast, in the present study, the use of chicory root in the diet led to decreased levels of androstenone in adipose tissue while maintaining unaltered levels of testosterone and IGF-1, and thus an intact anabolic potential. This suggests that chicory root in the diet can be used to selectively reduce androstenone without negative effect on pig growth.

Taken together this indicates that the differences in androstenone levels found between the two groups of pigs are due to increased androstenone catabolism and not anabolism, given the differences in 3β-HSD, but no difference in testosterone. The levels of testosterone and androstenone are strongly correlated [23,40], indicating the there was no difference in the androstenone anabolism in the two groups of pigs.

A well documented effect of dietary supplementation with chicory root is a decrease in the skatole production and accumulation [41]. In the present study we found no difference in skatole concentrations in plasma and adipose tissue between control and chicory root fed pigs. This is to some extent surprising, because we have previously shown that skatole metabolizing enzymes (CYP1A2, 2A and 2E1) in the liver are up-regulated when pigs are fed with chicory root [18]. Thus, it could be expected that skatole will be metabolized faster and less skatole will be available to accumulate in fat. This finding can be explained by the fact that skatole concentrations in this experiment were very low even in control pigs and thus they might not be genetically inclined to absorb high levels of skatole into the blood stream and adipose tissue. Alternatively, the low skatole levels in the pigs might be explained by a high hygienic environment [42]. In contrast, indole levels in the adipose tissue were greater in the chicory fed pigs. It is generally accepted that skatole and indole are regulated by a similar mechanism due to the common precursor [43] and a similar enzymatic system involved in the metabolism of both compounds [3,44]. Thus, it is expected that dietary manipulations would affect both skatole and indole in a similar way. However, a previous study demonstrated that this is not always true. Chen et al. [28] found that a dietary supplement of raw potato starch reduced skatole, but not indole levels in fat. Furthermore, addition of fructooligosaccharide to pig fecal slurries significantly reduced skatole but not indole synthesis from tryptophan [45]. Thus, taken together these *in vivo* and *in vitro* results imply that the regulation of skatole and indole in porcine tissues might differ. More studies are needed to investigate the underlying mechanism of these differences.

To evaluate if chicory root in the diet increased the cellular stress of the liver, the expression of HSP70 was analyzed. HSP70 is up-regulated when cells are stressed by heat, xenobiotics, *etc.* [19,20] and is often used as a marker of cellular stress. The HSP70 mRNA and protein expressions in the chicory fed pigs were not increased compared to control pigs. This indicates that the effects found in the present study and the effects found by Rasmussen et al. [18] are not due to increased hepatic stress.

5. Conclusion

The present study showed that feeding dried chicory root to male pigs for 16 days prior to slaughter reduces the concentration of androstenone in adipose tissue while the concentrations of testosterone and IGF-1 remained unaltered. We demonstrated that the reduction in androstenone levels is likely due to increased mRNA and protein expression of 3β-HSD, while SULT2A1 was not involved in this reduction. The present changes were not due to increased cellular stress as reflected by the level of HSP 70. Indole levels in fat were increased in the pigs fed chicory root, whereas skatole levels remained unaltered.

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Paper V

Dried chicory root modifies the activity and expression of hepatic CYP3A but not 2C – effect of *in vivo* and *in vitro* exposure

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Dried chicory root modifies the activity and expression of porcine hepatic CYP3A but not 2C - effect of *in vitro* and *in vivo* exposure

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Abstract

Hepatic cytochrome P450 expression and activity are dependent on many factors, including dietary ingredients. In the present study, feeding pigs with dried chicory root increased the expression of CYP3A29 mRNA but not of CYP2C33. Correspondingly, CYP3A activity was increased in pigs fed chicory root, while CYP2C activity was not affected. Additionally, the *in vitro* effect of chicory extract on the CYP3A activity was investigated. It was shown that CYP3A activity in the microsomes from male pigs was inhibited by chicory extract, but this effect was eliminated by pre-incubation. In both male and female pigs the CYP3A activity was increased in the presence of the chicory extract after pre-incubation. Furthermore, gender-related differences in mRNA expression and activity were observed. The CYP3A mRNA expression was greater in female pigs; however this was not reflected on CYP3A activity. For CYP2C, no difference in mRNA expression was observed, while CYP2C activity was greater in female pigs. Surprisingly, the expression of the constitutive androstane receptor, pregnane X receptor and aryl hydrocarbon receptor did not differ with feed or gender. In conclusion, chicory root modifies the expression and activity of CYP3A *in vivo* and *in vitro*, while CYP2C is not affected.

Keywords: Detoxification, Bioactive compounds, Phase I metabolism, Boar taint, Gender, Chicory

1. Introduction

The hepatic cytochrome P450s (CYP450) is a family of enzymes, important for the Phase I metabolism of xenobiotics and endogenous compounds. Many factors are involved in the regulation of CYP450 activity such as age, genetic background, health status and environmental factors. Additionally, it has been shown that expression and activity of some hepatic CYP450s can be up- or down-regulated by bioactive compounds from plants (Chang 2009; Delgoda and Westlake 2004; Nowack 2008; Zhou et al. 2003). An *in vitro* experiment using human hepatocytes showed that CYP2C19 activity was up-regulated upon treatment with extracts of St. John's Wort, Common Valerian, *Ginkgo biloba* and Common Sage (Hellum *et al.* 2009). Likewise, it has been shown using human hepatocytes that St. John's Wort induces CYP3A4 expression through activation of the pregnane X receptor (PXR) (Moore *et al.* 2000). Taken together, this shows that bioactive ingredients in the diet can act as ligands for the receptors regulating CYP450 expression, and thereby modify CYP450 expression and activity. However, regulation of CYP450 genes via its receptors is not straight forward, due to sharing of ligands and receptor cross-talk (Lewis 2004; Pascussi *et al.* 2008). Moreover, interpretation from one species to another is complicated by intra-species differences in CYP450 gene regulation (Martignoni *et al.* 2006). Apart from the *in vivo* effect of diet, previous studies using *in vitro* models have shown that bioactive plant compounds also modify the activity of CYP450s on the kinetic level, e.g. by competitive inhibition (Doehmer et al. 2011; Obermeier et al. 1995; Teel and Huynh 1998). Thus, introduction of bioactive compounds via feed can alter CYP450 activity, which in turn can affect the biological activity of drugs, endogenous hormones and other pathways of physiological importance.

The CYP3A and CYP2C isoforms are well studied in humans due to their involvement in the metabolism of a variety of drugs and endogenous compounds, including arachidonic acid, eicosanoids, progesterone, oestradiol, testosterone and cortisol (Lewis 2004; Nebert and Russell 2002; Waxman 1996). Together with other factors, diet is a critical player in the regulation of these isoforms in humans. However, knowledge about dietary dependent regulation of porcine CYP3A and CYP2C is missing.

The addition of chicory in the diet to pigs has been suggested as an alternative to castration to prevent the occurrence of boar taint, an unpleasant odour in meat from male pigs. For the successful implementation of chicory enriched diet in entire male pig production the side effects on liver metabolism have to be investigated in order to evaluate the safety of dietary chicory. Therefore, it is essential to investigate the hepatic expression and activity of CYP450 isoforms in the presence of chicory. Moreover, to evaluate the changes in CYP450 expression, the differences in constitutive expression of CYP3A and 2C between male and female pigs were investigated.

We have recently showed that feeding chicory root to pigs caused an increase in the expression and activities of CYP1A2 and 2A (Rasmussen et al. 2011e), as well as other hepatic enzymes (Rasmussen *et al.* 2012). In the present study, we investigated the *in vivo* and *in vitro* effect of chicory root on hepatic CYP3A and 2C. After 16 days of oral administration of chicory root to pigs we analysed the hepatic CYP450 mRNA expression and activity. Moreover the mRNA expression of the receptors regulating CYP450 expression was investigated. The *in vitro* effect of chicory on CYP450 activity was investigated in liver microsomes using specific probe substrates and a crude extract of chicory root.

2. Materials and Methods

2.1 Chemicals

All substrates and chemicals used were purchased for Sigma-Aldrich and of highest purity available. Primers and probes were custom made by DNA Technology (Aarhus, Denmark).

2.2 In vivo treatment and sample handling

The description of experimental animals, feed composition and slaughter procedure is given in Rasmussen *et al* (2011e). In brief: a group of male pigs were randomly allocated to either control (n = 6) group or experimental feed pigs (n = 14) 16 days before slaughter. Experimental feed pigs were fed energy matched diet containing 10 % dried chicory root for the 16 days before slaughter. Likewise a group (n = 4) of female pigs receiving control feed was included in the experiment. At an age of 164 days all pigs were slaughtered and liver samples were taken and stored at – 80 °C until further analysis. Microsomes for activity measurements were prepared according to (Rasmussen *et al.* 2011a) using sucrose-buffer and ultracentrifugation.

2.3 mRNA expression

Total RNA isolation and reverse transcription were done according to Rasmussen *et al* (2011c). Briefly, total RNA was isolated from frozen liver tissue using a Spin column according to the manufacturer's instructions (Rnasey Mini Kit, WVR, Herlev, Denmark). Equal amounts of RNA was converted to cDNA using SuperScript II Rnase H Reverse Transcriptase and Oligo(dT)12-18 Primer (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. Real-time polymerase chain reaction (PCR) and designing of primers and probes were done according to Rasmussen *et al* (2011b; 2011c) with the use of porcine specific sequences of genomic DNA (http://www.ensembl.org/Sus_scrofa/Info/Index). Primers and probes are given in Table 1. All samples were analysed in duplicates in a 348 well plates using a ABI 7900 HT sequence detection system (Applied Biosystems, Carlsbad, CA, USA). The PCR was conducted under the following conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 min.

Relative mRNA expression was calculated by relating threshold cycles to a standard curve obtained by running a serial dilution of a cDNA sample. Relative mRNA expression was normalised against the mRNA expression of GAPDH. The average of control samples was arbitrary set to 1. The expression of GAPDH was not significantly affected by the treatment.

2.4 Activities of CYP3A and 2C

The activities of 7-benzyloxyresorufin O-dealkylase (BROD; CYP3A), 7-benzyloxyquinoline O-debenzylase (BQOD; CYP3A) and tolbutamide hydroxylase (TBOH; CYP2C) were measured. Validation of specificity and analytical procedures for porcine BROD and BQOD were described by Zlabek and Zamaratskaia (2011) and for tolbutamide by Skaanild and Friis (2008)Zamaratskaia *et al*. (2012).Briefly, to measure BROD and BQOD activity, the incubation mixtures (0.5 ml) contained 0.2 mg of microsomal protein in 0.5 M potassium phosphate buffer (pH 7.4), appropriate substrate (100 µM of BQ or 4 µM of BR) and 0.5 mM of NADPH. The microsomes and substrates were pre-incubated for 2 min prior to start of the reaction by addition of NADPH. The mixtures were incubated in a water bath at 37 °C for 10 min and the reactions were terminated with ice-cold 100% methanol (500 µL). To measure TBOH activity, the incubation mixtures (0.25 ml) contained 0.5 mg of microsomal protein in 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA buffer (pH 7.4), 200 µM of tolbutamide and 1 mM of NADPH. The mixtures were incubated in a water bath at 37 °C for 40 min without pre-incubation and the reactions were terminated with the addition of 0.25 ml of 100% cold acetonitrile.

2.5 In vitro inhibition study

The positive control for enzyme inhibition of BROD and BQOD by selective inhibitors has previously reported (Zlabek and Zamaratskaia 2011). Direct effect of chicory root extract (CRE) on the activities of BROD and BQOD was investigated using microsomal preparations from male and female pigs. The pigs used for preparation of the used microsomes have not previously been exposed to chicory. CRE was prepared by mixing chopped dried chicory root with 96 % ethanol in 1:1 for 24 h, followed by filtration. The resulting liquid phase was used as a crude extract of chicory. Three dilutions of CRE were used (0, 10 and 100 times dilutions). In each experimental set 3 pigs of each

gender were included. Activities were determined with or without a 5 min pre-incubation step of the microsomes and CRE (+ NADPH), before addition of the probe substrate. The pre-incubation step was introduced to test for the effect of metabolites produced from the CRE. Results are presented as percentage of activity in absence of CRE, but in the presence of < 0.1 % ethanol (control samples). Preliminary analysis confirmed that the activity was not affected by the presence of < 0.1% ethanol (data not shown). All incubations were performed in duplicate.

2.6 Statistics

In the *in vivo* experiment groups was compared using Student's unpaired t-test. Data from the *in vitro* study were statistically evaluated using two-way ANOVA (SAS version 9.2; SAS Institute, Cary, NC, USA). The model included CRE dilution and gender as fixed factors, and individual microsomes pools incubation as a random factor. Likewise, interaction between CRE dilution and gender was also included in the model. Analyses were performed separately with or without the pre-incubation step. The differences were considered significant at $P < 0.05$.

3. Results

3.1 In vivo effect of chicory on CYP expression and activity

In human CYP3A4 and 2C9 isoforms of the CYP3A and 2C families has been shown to the most important once for the metabolism of drugs (Guengerich 2007). The porcine versions of these isoforms, based on amino acid sequence match are CYP3A29/3A39 and CYP2C33, respectively (Achour et al. 2011). For CYP3A29 vs 3A39, 3A29 has been shown to most resemble the properties of the human CYP3A4 (Puccinelli et al. 2011).

In pigs fed dried chicory root, the mRNA expression of CYP3A29 was 2.0 fold greater ($p < 0.05$) than in control feed male pigs, while no difference was observed in CYP2C33 mRNA expression (Figure 1). The CYP3A dependent activity was determined using two different reactions, BROD and BQOD (Table 2). In chicory feed pigs the BROD and BQOD was 2.0 and 3.1 times greater than in control feed pigs, respectively ($p < 0.05$). For the CYP2C dependent activity determined by TBOH there was 4.0 times greater activity in chicory feed pigs. However, due to very large inter-individual variation this difference was not significant ($p = 0.07$). To investigate the underlying mechanism for the difference in CYP450 expression, expression of the receptors regulating CYP450 expression was investigated. No differences in the expression of the constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AhR) or PXR were observed between chicory and control feed pigs (Figure 2).

3.2 Gender-related difference in CYP3A and 2C

The gender-related difference in constitutive CYP3A29 and 2C33 mRNA expression and activity was investigated (Figure 1). The mRNA expression of CYP3A29 was 2.1 fold greater in female pigs compared to control feed male pigs ($p < 0.05$), while the CYP2C33 expression did not differ. Surprisingly, corresponding differences in CYP3A or 2C dependent activity were not observed (Table 2). In female pigs the TBOH was 1.3 times greater compared to control ($p < 0.05$), while the CYP3A dependent activity did not differ with gender. There was no difference in the constitutive mRNA expression of CAR, AhR and PXR between genders (Figure 2).

3.3 In vitro effect of CRE on BROD and BQOD activities

Both BROD and BQOD activities in the microsomes from male pigs were decreased in the presence of undiluted CRE (Table 3). This decrease was eliminated by the addition of the pre-incubation step. Moreover, BROD but not BQOD activity was increased in the presence of 10 and 100 times diluted CRE when the pre-incubation step was included. In the microsomes from female pigs, BROD activity was significantly decreased in the presence of undiluted CRE without pre-incubation. Interestingly, no inhibition of BQOD activity was observed either with or without pre-incubation in the microsomes from female pigs (Table 3). In the microsomes from female pigs, the BQOD was increased by the presence of undiluted CRE, while including the pre-incubation step, only the 100 times diluted CRE increased the activity.

4. Discussion

The hepatic CYP450 activity can be regulated by many different events, including *1)* up-regulation of gene expression, *2)* mRNA translation, *3)* protein stabilization and *4)* inhibition or activation of the catalytic activity of the enzyme. Regulation of CYP450s has been extensively studied in humans, rats and mice, while porcine CYP450 regulation has received less attention. Knowledge on the possible interaction of feed ingredients with porcine CYP450s is valuable for pig production and because pigs have been suggested as models for humans (Puccinelli *et al.* 2011). Moreover, exposure to novel feed ingredient that modify CYP450 dependent metabolism can lead to subsequent unwanted side effects, when taking into account the simultaneously administration of drugs. For example, the modulation of CYP3A and CYP2C can have unpredictable physiological consequences as both enzymes account for metabolism of many drugs as well as endogenous compounds. The use of chicory root as a feed supplement to pigs has recently attracted considerable attention because of its positive impact on meat quality, particularly by the reduction of skatole levels, which together with androstenone contributes to the development of boar taint (Zamaratskaia and Squires 2009). This effect is partly believed to be due to a pre-biotic effect of inulin, a major component of chicory root. However, there are a number of bioactive secondary metabolites in chicory, like sesquiterpene lactones (Bais and Ravishankar 2001). Artemisinin (a sesquiterpene lactone from *Artemisia* sp.) has been shown to up-regulate CYP3A4 and CYP2B6 in humans and mice by binding to the nuclear receptors PXR and CAR (Burk *et al.* 2005). A study from our lab has shown that feeding chicory to pigs increases the mRNA expression of CYP1A2, CYP2A and CYP2E1, as well as protein expression and activity of CYP1A2 and CYP2A (Rasmussen et al. 2011e). In the same pigs, the present study showed that feeding chicory root caused a significant increase in expression of CYP3A29 mRNA and activities of BROD and BQOD. Additionally the expression of CYP2C33 mRNA and TBOH activity remained unchanged. The mRNA expression of CYP450s is commonly believed to be regulated by receptors, like AhR, CAR and PXR. Thus, to further investigate the mechanism behind the increase in CYP450 mRNA with chicory, we investigated the mRNA expression of these receptors. The expression of the investigated nuclear receptors was not changed by feeding chicory root. This suggests that regulation of CYP3A and other

CYP450s by chicory root is more complex and does not involve a direct coupling to changes in nuclear receptor expression, but could be the action of ligands activating the receptors.

Direct effects of phytochemicals on CYP450 dependent activity has been shown (Doehmer et al. 2011; Obermeier et al. 1995; Teel and Huynh 1998). Scott *et al* (2006) showed that *in vitro* CYP3A4, CYP19 and CYP2C19 activities could be modified by various plant constituents, and that the magnitude of inhibition is dependent upon the concentration of bioactive constituents in the extract. Thus, to further investigate the impact of chicory root on CYP3A dependent activity, the direct effect of CRE on BROD and BQOD was investigated. Both BROD and BQOD activities in microsomes from male pigs were decreased by the presence of undiluted CRE. This decrease in activity was eliminated when diluted CRE was used.

For many of the CYP450 *in vivo* activities, a non-Michaelis Menten kinetics or mechanism based inhibition is reported (Atkins 2004; Venkatakrishnan et al. 2001; Zhang and Wong 2005). We have in earlier studies found that pre-incubation of steroids is important for the study of *in vitro* CYP activity (Rasmussen et al. 2011d; Zamaratskaia et al. 2007). In order to study this, a pre-incubation step was included, to imitate the situation *in vivo*. Interestingly, an increase in BROD and BQOD activities by CRE was observed when a pre-incubation step was included. This might be explained by the formation of metabolites from some parent CRE components, acting as stimulator of CYP3A activity.

Overall, the *in vitro* effect of CRE on BROD and BQOD differed in microsomes from male and female pigs. This is in agreement with our previous study (Rasmussen *et al.* 2011c), where we suggested gender-dependent differences in the mechanism regulating CYP450 dependent activity. These findings show that it is important that both genders are included when conducting studies on the regulation of CYP450 by dietary ingredients. The effect of CRE on CYP activity in microsomes from the chicory fed male pigs, was not determined because they might have been adapted to the challenge of the compounds present in chicory during the feeding time. For that reason the acute effect of chicory exposure cannot be determined. The effect of CRE on TBOH *in vitro* was not investigated due to the lack of effect of chicory on TBOH *in vivo*.

Gender-dependent differences in porcine hepatic CYP450 expression and activity are well known (Kojima et al. 2008; Rasmussen et al. 2011c; Skaanild and Friis 1999; Zamaratskaia et al. 2006). However, to our knowledge the role of gender in CYP3A and 2C expression and activity in pigs has not been completely elucidated. The present study shows that female pigs have greater constitutive expression of CYP3A29 mRNA, while no differences were observed when comparing BROD or BQOD between genders. For CYP2C33, no differences between mRNA expression was observed, while female pigs showed greater TBOD activity. The lower CYP3A29 mRNA expression in male pig is probably due the presence of some testicular steroids, absent in female pigs, as studies have shown that castration of male pigs increases CYP3A expression and activity (Gillberg et al. 2006; Niemelä et al. 1999). It should be noticed that the present study has not fully coveraged the whole CYP3A and 2C family with respect to mRNA expression and that this can explain the discrepancies between mRNA expression and activity measurements. For CYP2C the present study shows no difference in mRNA expression, while the activity was greater in female pigs. It has previously been suggested that male pigs have greater CYP2C activity than female pigs (Skaanild and Friis 2008). This finding is opposite to what the present study shows and what is the general trend in gender-dependent expression, with female having greater CYP450 expression and activity. The discrepancies between mRNA expression and activity between gender and the contradictory results on CYP2C need to be addressed in further studies.

5. Conclusion

This is the first study to show that feeding dried chicory root to pigs induced the mRNA expression and increased the activity of CYP3A. These changes were not directly linked to changes in PXR expression. The exact mechanism of the regulation of CYP3A by chicory root is yet to be elucidated. The mRNA expression and activity of CYP2C were not affected by feeding dried chicory root.

Conflict of Interest Statements

The authors declare that there are no conflicts of interest.

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Tables

Table 1. Primers and TaqMan probes for PCR.

Tabel 2. BROD and BQOD activity (pmol/min/mg total protein) in microsomes from male/control (n = 6), female (n = 4) and chicory feed male (n = 14) pigs.

BROD, 7-benzyloxyresorufin O-dealkylase; BQOD, 7-benzyloxyquinoline O-debenzylase; TBOH, tolbutamide hydroxylase. Data are presented as mean values ± standard deviation. * Significantly different from male/control group ($p < 0.05$)

Table 3. *In* vitro effect of crude chicory extract (CRE) on CYP3A dependent activities in porcine liver microsomes.

Data are presented as percentage of remaining activity ± standard deviation. BROD, 7-benzyloxyresorufin O-dealkylation; BQOD, 7-benzyloxyquinoline O-debenzylation. Levels of significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (different from the control incubations without addition of chicory root extract (CRE))

Figures

Figure 1. Expression of porcine cytochrome P450 isoform 3A29 and 2C33 mRNA in male pigs feed chicory root for 16 days; and in relation to gender. Values are mean ± standard deviation relative to the male/control group. * Significantly different from male/control group ($p < 0.05$)

Figure 2. Expression of porcine constitutive androstane receptor (CAR), pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR) in male pigs feed chicory root for

16 days; and in relation to gender. Values are mean ± standard deviation relative to the male/control group.

Table 1.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	TaqMan probe (5'-3')	Reference
CYP3A29	GGACACCATAAATCCTTACACTTACCT	GCAAACCTCATGCCAATGC	CCTTTGGGACTGGACCCCGCAA	(Nannelli et al. 2010)
CYP2C33	TGGGAATCTGATGCAACTTAACC	AACAGGGCCGTACTGTTTGG	AAGGACATCCCTGCGTCTCTTTCCAAGTT	(Tomankova et al. 2012)
AhR	AGCTGCACTGGGCGTTAAACT	GCCACTCGCTTCATCAATTCT	CCTTCACAGTGTCCAGACTCTGGAC	(Messina et al. 2009)
CAR	TTCATCCATCACCAGCACTTG	TGATGTCCGCGAAATGCA	CCCTGGTGCCTGAACTGTCTCTGCTC	(Nannelli et al. 2010)
PXR	GCTGAACTGTGCTAGGCTTCTG	CCTCCCACGAGCCATGTT	ATGCACCGGGACACAAGTGAGGG	(Nannelli et al. 2010)
GAPDH	GTCGGAGTGAACGGATTTGG	CAATGTCCACTTTGCCAGAGTTAA	CGCCTGGTCACCAGGGCTGCT	(Young et al. 2008)

Table 2.

	Male/Control	Female	Male/chicory
BROD	6.6 ± 3.6	4.2 ± 0.9	13.6 ± 7.5 *
BQOD	297.1 ± 185.9	375.9 ± 143.3	919.9 ± 550.1 *
TBOH	26.0 ± 10.2	40.4 ± 4.5 *	104.7 ± 97.1

Table 3

	CRE dilution	BROD, remaining activity, %		BQOD, remaining activity, %	
Sex		Male	Female	Male	Female
No pre-incubation	0 times	70 ± 5.4***	80 ± 5.4*	59 ± 13.9*	147 ± 13.9*
	10 times	101 ± 5.4	90 ± 5.4	95 ± 13.9	135 ± 13.9
	100 times	104 ± 5.4	95 ± 5.4	85 ± 13.9	122 ± 13.9
Pre-incubation 5 min	0 times	85 ± 14.0	83 ± 14.0	97 ± 17.1	126 ± 17.1
	10 times	154 ± 14.0**	90 ± 14.0	68 ± 17.1	124 ± 17.1
	100 times	169 ± 14.0 ***	106 ± 14.0	84 ± 17.1	155 ± 17.1*

Figure 1

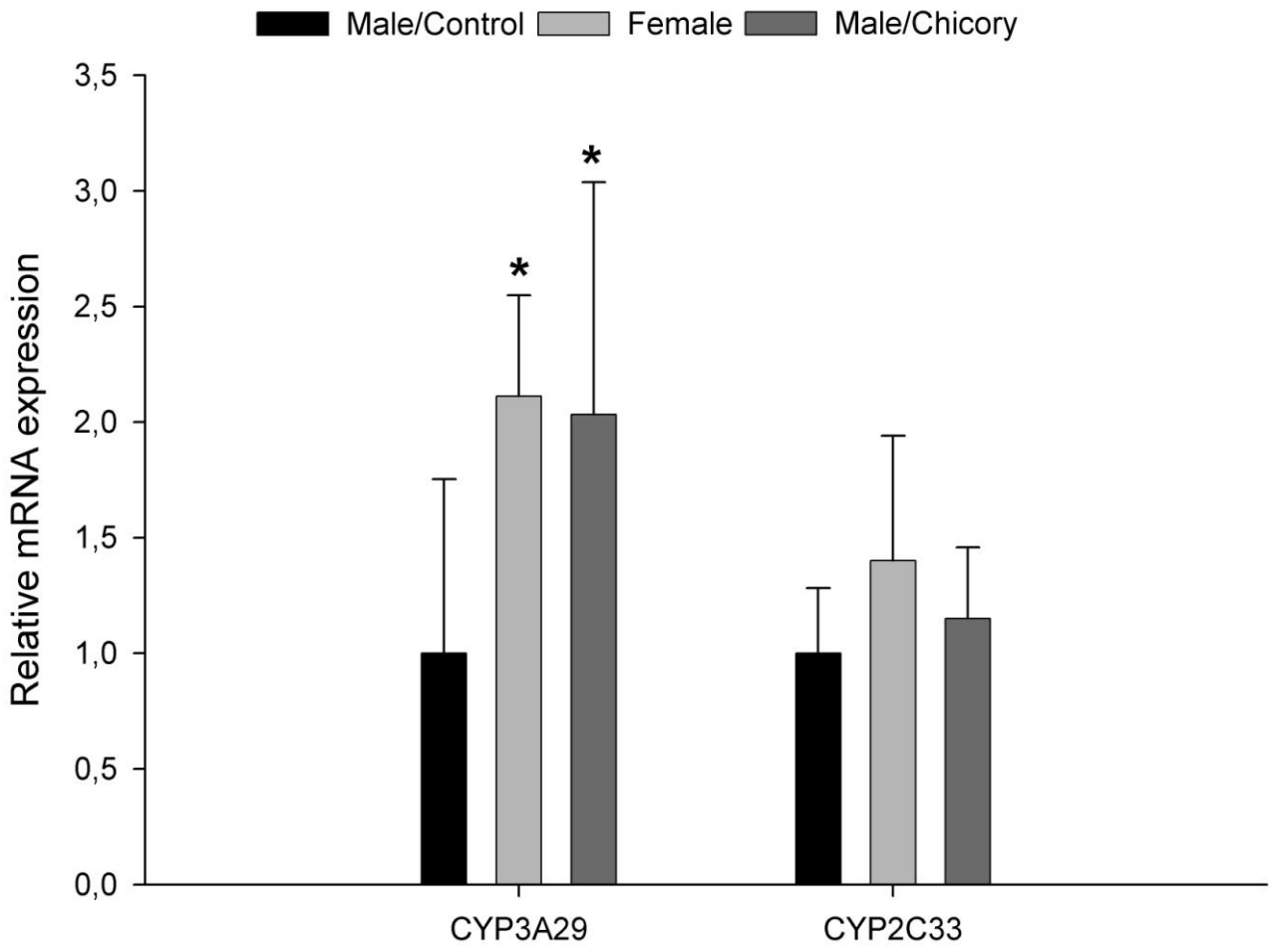
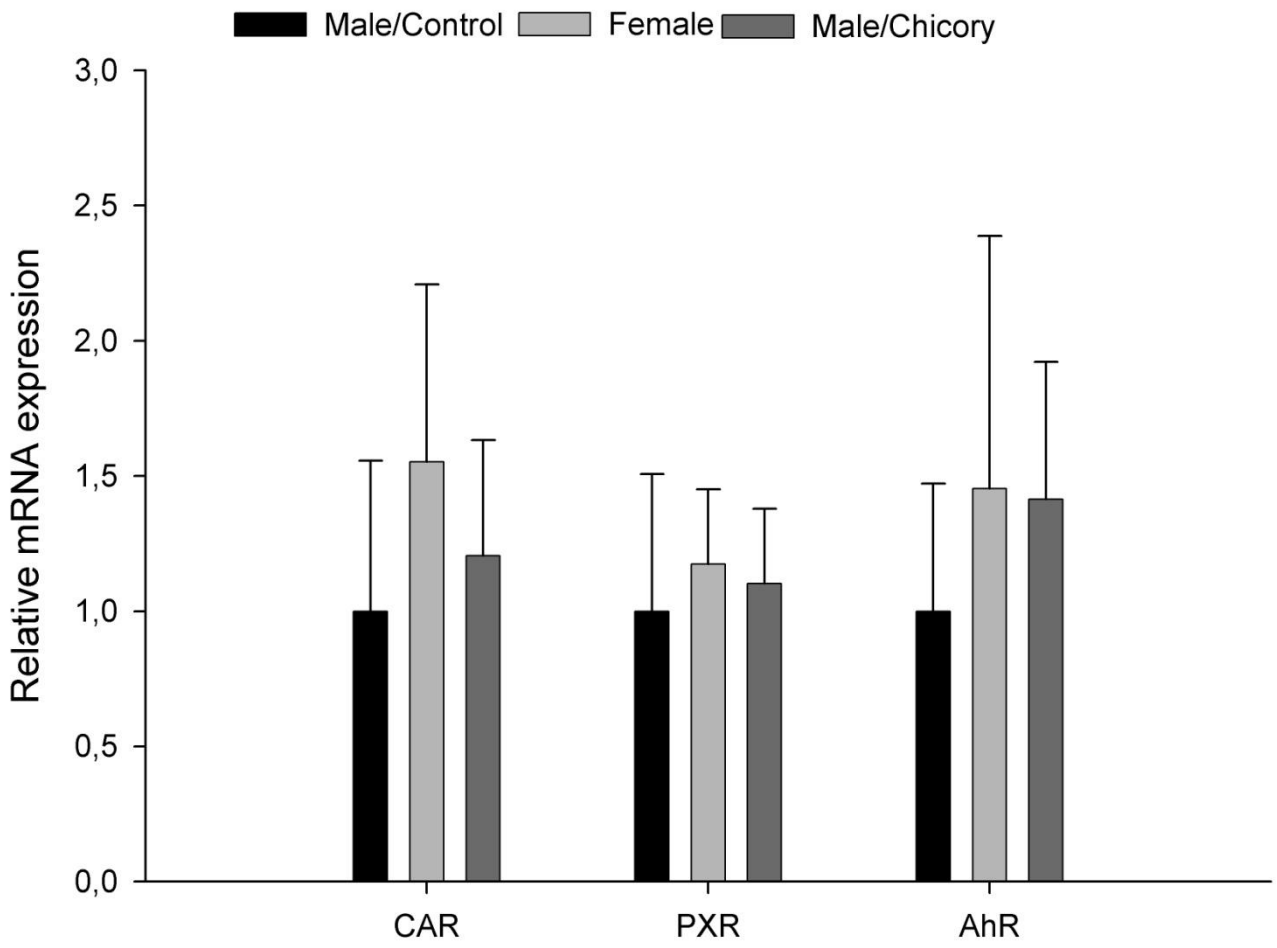

Male/Control
Female
Male/Chicory
Relative mRNA expression
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
*
CYP3A29
CYP2C33

Figure 2


Male/Control
Female
Male/Chicory
Relative mRNA expression
3,0
2,5
2,0
1,5
1,0
0,5
0,0
CAR
PXR
AhR

Paper VI

Chicory root (*Cichorium intybus L.*) extract down-regulates cytochrome P450 mRNA expression in porcine hepatocytes

Martin Krøyer Rasmussen, Christina Lindgaard Jensen and Bo Ekstrand

Submitted to Drug Metabolism and Disposition

Chicory root (*Cichorium intybus* L.) extract down-regulates cytochrome P450 mRNA expression in porcine hepatocytes

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Running title: *In vitro* regulation of Porcine CYP450 mRNA by chicory

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Abbreviation list:

CYP, cytochrome P450 peroxidase; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; PXR, pregnane X receptor; CITCO, 6-(4chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene

Abstract

Chicory (*Cichorium intybus L.*) possesses several pharmacological properties, among them hepatoprotective effects. Additionally, chicory has been shown to induce pharmacologically relevant enzymes (cytochrome P450; CYP) *in vivo*. The aim of the study was to evaluate the effect of a methanolic extract of dried chicory root on hepatic CYP (1A2, 2A19, 2C33, 2D25, 2E1 and 3A29) mRNA expression *in vitro*. Primary porcine hepatocytes were isolated from female piglets and treated with various amounts of chicory extract. After 24 hours of treatment, hepatocytes were harvested and specific CYP mRNA expression determined by PCR. Additionally the effects of chicory were compared to that of standard CYP inducers as well as secondary plant metabolites (artemisinin and scoparone). Chicory root extract decreased the mRNA expression of CYP1A2, 2C33, 2D25 and 3A29 when high concentrations were used. Low concentrations of chicory extract had no effect of CYP expression. No effect of chicory on CYP2A19 and 2E1 was shown. The effect of chicory was opposite to that of standard CYP inducers and artemisinin and scoparone. In conclusion, a methanolic extract of chicory root down-regulates CYP mRNA expression *in vitro*. These results contribute to the understanding of the pharmacological properties of chicory. Moreover they suggest that one or more compounds in chicory can down-regulate cytochrome P450 expression and thereby act as hepatoprotective agents.

Introduction

Chicory (*Cichorium intybus L.*) has been shown to possess hepatoprotective effects and is part of a common Indian polyherbal drug (*Liv 52*) (Ahmed *et al.* 2003; Bais and Ravishankar 2001; Gadgoli and Mishra 1997). Additionally, studies have shown anti-inflammatory, anti-malarial and anti-diabetic properties of phytochemicals isolated from chicory (Cavin, 2005; Tousch, 2008; Bischoff, 2004). Moreover, chicory has previously been shown to increase expression and activity of hepatic drug metabolizing enzymes in pigs *in vivo* (Rasmussen *et al.* 2011d).

The liver is the single most important organ in the metabolism of xenobiotics as well as many endogenous compounds in the body. For studies of liver metabolism in general, *in vivo* experiments using whole animals has been extensively used, but there is a need for good and accurate *in vitro* models for high throughput screening to elucidate the molecular mechanisms of regulation. Different model systems including liver slices (Edwards *et al.* 2003), microsomes (Rasmussen *et al.* 2011a) and isolated hepatocytes (Monshouwer *et al.* 1998) have been used as models to investigate the effects of various compounds on important liver functions. For studying the gene regulation of hepatic enzymes, isolated hepatocytes is the method of choice, and much research has been done on their performance in cultures (reviewed by LeCluyse 2001).

Metabolism of the diverse classes of xenobiotics (drugs, antibiotics etc.) is usually divided into two phases: Phase I, oxidative modification, and Phase II, derivatisation. Phase I is often catalyzed by enzymes belonging to the cytochrome P450 peroxidase (CYP) superfamily. Hence, CYPs are extensively studied for their involvement in drug metabolism and detoxification (Guengerich 2007). In pigs the CYP2A, 2C, 2D, 2E and

3A families make up a total of 98 % of all CYPs on the protein level, with 2A19 and 2D25 being the most abundant (Achour *et al.* 2011). Apart from their impact on drug metabolism mentioned above, porcine CYPs are also studied for their ability to metabolize skatole. The compound skatole is a metabolite of tryptophan produced in the guts of pigs; after absorption to the blood stream it is metabolized in the liver by CYPs. If the hepatic clearance of skatole is insufficient, it will accumulate in the fat. High concentrations of skatole in fat are associated with poor meat quality of sexually mature male pigs, a phenomenon known as boar taint. Several different CYPs have been shown to catabolise skatole (Matal *et al.* 2009; Terner *et al.* 2006; Wiercinska *et al.* 2012). Differences in CYP expression and activity between individuals have been observed and are often explained by the genetic profile of the individual, but other factors are also important, like gender, age, etc. Moreover, there is growing evidence that bioactive compounds affect liver function and CYP activity. It has been shown that compounds in grapefruit juice inhibits the metabolism of drugs by affecting CYP activity (Kiani and Imam 2007). Likewise, St. John's Wort (*Hypericum*), *Ginkgo biloba*, Wormwood (*Artemisia),* Common sage (*Salvia)* and Chicory (*Cichorium*) have been shown to influence the expression and/or activity of hepatic CYPs (Chang 2009; Chang and Waxman 2006; Hellum *et al.* 2009; Rasmussen *et al.* 2011d). Thus, the aim of this study was to investigate the effect of chicory and secondary plant metabolites on CYP mRNA expression *in vitro*.

The expression of CYPs is generally believed to be regulated by xenobiotic receptors like the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR), regulating CYP1A, 2A and 3A expression respectively. However extensive cross-talk between receptors does take place (Reviewed by Pascussi

et al. 2004; 2008). By treating hepatocytes with standard activators of these receptors the potential for CYP mRNA induction can be investigated. The effect of chicory root extract was compared to that of the standard CYP inducers.

Materials and methods

Ethical and animal welfare aspects

The pigs used for hepatocyte isolation were treated in accordance with the guidelines from the Danish Inspectorate of Animal Experimentation.

Isolation of hepatocytes

Five female cross bred (Landrace x Yorkshire sire and Duroc boar) piglets (11.1 ± 0.5 kg) were used in this study. The pigs were killed with a bolt pistol followed by immediate exsanguination, before the liver (weight 200 - 250 g) was removed and transported on ice to the laboratory. Time from killing to the arrival of the liver at the laboratory was maximum 10 minutes. Hepatocytes were isolated by a protocol adopted from Monshouwer *et al* (1996) and Seger (1972). After arrival at the laboratory the liver was transferred to a laminar flow-bench and cannulated with a tube in the large central vein. The liver was then perfused with 1 L of basis-buffer (Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (DPBS; Invitrogen) supplemented with 10 mM HEPES, 1 % glucose; saturated with O_2; pH 7.4) added 1 mM EGTA, followed by perfusion with 1 L basis-buffer. Afterwards 0.75 L basis-buffer containing 4.75 mM $CaCl_2$ and 0.05 % collagenase (Type II; Worthington, USA) was perfused under recirculation for 15 min. The liver was then cut into small pieces with a scalpel and transferred to a bottle containing basis-buffer with 4.75 mM $CaCl_2$ and 0.025 % collagenase and incubated under gentle stirring for 15 min. To the resulting cell suspension ice-cold basis-buffer with 2 % BSA in 1:1 was added, and it was then filtered through nylon mesh with a mesh size of 200 μm^2 followed by filtering through nylon mesh with a mesh size of 50 μm^2. Cells were then isolated by a 5 min

centrifugation at 100 g (4 °C) and washed in William E medium (WME; Invitrogen) containing 10 % (volume/volume) foetal calf serum (FCS; Sigma). After isolation of the cells, they were seeded directly or cryopreserved according to the procedure described below.

Cell number, viability, cryopreservation and thawing of hepatocytes

Cell number was evaluated by counting the cells in a haemocytometer under a microscope. Cell viability was estimated by their ability to exclude trypan blue, regarding cells not able to exclude trypan blue as dead.

The isolated cells were diluted in WME with 10 % FCS, 2 mM L-glutamine (Sigma) and 1 µM insulin (Sigma) obtaining a final density of 10^7 cells pr ml. DMSO was slowly added to the cell suspension reaching a final concentration of 10 % (v/v). Afterwards 1.0 ml cell suspension was put in cryotubes (NUNC) and frozen in propanol containing boxes, giving a 1 °C drop in temperature pr min until – 80 °C was reached. The next day tubes were transferred to a tank containing liquid nitrogen.

Thawing was done by heating the tubes in a 37 °C water bath and, when the content was thawed, it was transferred to 50 ml tubes with WME containing 10 % FCS (37 °C). The cells were then sedimented by centrifugation at 100 g for 5 min (37 °C).

Effect of time in culture

For evaluation of the effect of cryopreservation and time in culture, either freshly isolated or cryopreserved hepatocytes were suspended in WME with 10 % FCS, 2 mM L-glutamine, 100 unit/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 20 µg/ml gentamicin (Sigma), 2.5 µg/ml ampotericin B (Sigma) and 1 µM insulin. 100.000 cells

pr cm^2 were seeded into collagen (Type I, BD Biosciences) coated wells (6 well plates; NUNC) and subjected to 37 °C and 5 % CO_2 in an incubator. 24 hours after seeding, dead and unattached cells were removed by washing the wells with DPBS (+ Ca^{2+}, Mg^{2}+; 37 °C) and fresh medium was added. This time point was regarded as day 1. Medium was renewed every 24 h and samples collected equivalent on day 2, 3, 6 and 7.

Induction

To test the functionality of the isolated hepatocytes we determined their response to standard CYP inducers. We used 100 µM β-naphtoflavone (Sigma), 100 µM CITCO (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime; Sigma), 50 µM dexamethasone (Sigma) and 5 nM TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene; Sigma) dissolved in DMSO giving a final concentration in the media of 0.1 % DMSO. Likewise, 1-100 µM artemisinin (a sesquiterpene lactone, Mediplantex, Hanoi, Vietnam) and 10-100 µM scoparone (6,7-dimethoxycoumarin; Sigma) were dissolved in DMSO giving a final concentration of 0.1 % DMSO in the media.

A crude extract of dried chicory root was made by mixing 1:3 (w/v) of dried chicory root to 96 % methanol. After 24 h of extraction, the extract was filtered and the methanol removed under vacuum, resulting in a 50 times reduction in volume. The final extract was given an arbitrary concentration of 100.000 units. For all added concentration of extract, methanol was added to the media, giving a final concentration of methanol at 0.1 %.

Cryopreserved hepatocytes were seeded at a density of 100.000 cells pr cm^2 into 12 well collagen coated plastic plates. The cells were seeded in WME with 5 % FCS, 2 mM L-

glutamine, 100 unit/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamicin, 2.5 µg/ml ampotericin B and 1 µM insulin. After 24 hours of attachments the wells were washed with DPBS and fresh medium with or without inducers/extract was added for another 24 hours before the cells were lysed.

The control samples were added either 0.1 % DMSO or methanol. In preliminary experiments it was shown that DMSO and methanol at these concentrations did not affect the CYP mRNA expression (data not shown). All treatments were analysed in duplicates for each cell isolate.

Quantitative PCR

RNA isolation and reverse transcription were done according to Rasmussen *et al* (2011c). Briefly, total RNA was isolated by lysing the cell in the wells with RLT-buffer (WVR, Herlev, Denmark) and running the lysate on a Spin column according to the manufacturer's instructions (Rnasey Mini Kit, WVR, Herlev, Denmark). Equal volumes of RNA were converted to cDNA using SuperScript II Rnase H Reverse Transcriptase and Oligo(dT)12-18 Primer (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. Polymerase chain reaction (PCR) and designing of primers and probes were done according to Rasmussen *et al* (2011b; 2011c) with the use of porcine specific sequences of genomic DNA (http://www.ensembl.org/Sus_scrofa /Info/Index). Primers and probes for CYP1A2, 2A19, 2E1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are given in Rasmussen *et al* (2011c), while CYP3A29 is given in Nannelli *et al* (2010). For determination of CYP2C33 we used forward primer, reverse primer and TaqMan probes with the following sequences (5'-3'); TGGGAATCTGATGCAACTTAACC, AACAGGGCCGTACTGTTTGG and

AAGGACATCCCTGCGTCTCTTTCCAAGTT, respectively. For determination of CYP2D25 the corresponding sequences were; TTGGAAGGACTGAAGGAGGAAGT, CCCGGGATATGCCTGAGAA and CCTCATGCGCCAGGTGCTGGA. The samples were analysed in duplicates in 348 well plates using a ABI 7900 HT sequence detection system (Applied Biosystems, Carlsbad, CA, USA) under the following conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 min.

Relative mRNA expression was calculated by relating threshold cycles to a standard curve obtained by running a serial dilution of a cDNA sample. Relative mRNA expression was normalised against the mRNA expression of GAPDH. The average of control samples was arbitrary set to 1. The obtained Ct-values for GAPDH were not significantly affected by any of the treatments.

Statistical analysis

To evaluate the effect of cryopreservation and time in culture on CYP mRNA expression two-way ANOVA was used, followed by Tukeys post hoc test to identify differences in CYP mRNA expression. The effect of inducers, artemisinin and scoparone was evaluated by one-way ANOVA, followed by Tukeys post hoc test. Too evaluate the effect of the chicory root extract compared to the control samples, Student's t-test with Bonferroni's correction was used (a corrected p-level of $p < 0.0083$ was regarded as significant).

Results

Isolation of hepatocytes

The total yield of hepatocytes was between $2 \cdot 10^8$ to $5 \cdot 10^9$ cells. When comparing the morphology of freshly isolated and cryopreserved hepatocytes, no differences were seen, whereas the viability before seeding was lowered by cryopreservation ($p < 0.01$; Table 1). Due to low attachment of the cryopreserved hepatocytes isolated from one pig (Table 1; Pig B), a Percoll purification (Kreamer *et al.* 1986) was performed before seeding.

CYP mRNA expression in hepatocytes, effect of time in culture

For CYP1A2, 2A19, 2D25 and 2E1 there was a significant decrease in mRNA expression on day 2 in culture compared to day 1 (Figure 1). This reduction was maintained for the rest of the period. For CYP2C33 and 3A29 there was no significant difference in mRNA expression between the days in culture. However, for these CYPs large variations between the cell isolates were observed. For all CYPs investigated, no differences in mRNA expression between freshly isolated and cryopreserved hepatocytes in relation to their time in culture were observed.

Induction of CYP mRNA

To test the potential of the model to modify mRNA expression of the investigated CYPs, the hepatocytes were treated with standard activators of the receptors regulating gene expression. The used inducers were 100 µM β-naphtoflavone (figure 2A), 100 µM CITCO (figure 2B), 50 µM dexamethasone (figure 2C) or 5 nM TCPOBOP (figure 2D)

for 24 hours. Treating hepatocytes with β-naphtoflavone significantly increased the mRNA expression of CYP1A2 5.1 fold compared to control. Likewise the mRNA expression of CYP1A2 was significantly larger than the expression of all other investigated CYP isoforms. Treatment of hepatocytes with CITCO significantly increased the mRNA expression of CYP1A2 4.0 fold compared to control. Furthermore the mRNA expression of CYP1A2 was significantly larger than for all other investigated CYP isoforms. In the dexamethasone treated hepatocytes mRNA expression of CYP1A2 and 3A was significantly increased 4.9 and 5.3 fold, respectively, compared to control. While the 4.3 fold increase in CYP2C33 expression almost reached significance ($p = 0.054$). There was no effect on CYP mRNA expression of treating hepatocytes with TCPOBOP (Anova, $p > 0.05$).

Chicory extract, artemisinin and scoparone

Treatment of hepatocytes with artemisinin significantly increased the mRNA expression of CYP1A2, 2C33, 2D25, 2E1 and 3A29, up to 3.5, 1.5, 1.1 and 3.4 fold, respectively, compared to control (figure 3A). Scoparone significantly increased the mRNA expression of CYP1A2 and 2D25, up to 4.3 and 1.8 fold, respectively, compared to control (figure 3B). Apart from that, no other effects on CYP mRNA expression was observed.

The extract of chicory decreased the mRNA expression of CYP1A2 when administrated in the three highest concentrations (figure 4A). Moreover, chicory extract also decreased the mRNA expression of CYP2C33, 2D25 and 3A29 when administrated in the highest concentration (figure 4C, D, F). No effect off the chicory root extract was observed on CYP2A19 and 2E1 expression.

Discussion

The aim of the present study was to use isolated hepatocytes from piglets to investigate regulation of CYP mRNA expression by treatment with a methanol extract of chicory root. In order to validate the hepatocyte model, the effects of time in culture, standard CYP inducers and isolated secondary plant metabolites on CYP expression were investigated. The major findings were that 1) mRNA expression of the investigated CYPs with time in culture was not affected by cryopreservation; 2) treatment with standard CYP inducers and isolated plant secondary metabolites increased CYP expression whereas 3) chicory root extract lowered the expression of some of the investigated CYPs, but not all.

Several studies have investigated the effect of time in culture on overall and specific CYP protein expression and activity (Gillberg *et al.* 2006; Hg *et al.* 1995; Keatch *et al.* 2002; Loven *et al.* 2005; Monshouwer *et al.* 1998; Skaanild and Friis 2000; Zhou *et al.* 1998). To our knowledge this is the first study to investigate the effect of time in culture on mRNA expression, while the studies mentioned above all investigated the protein expression or activity. In the present study we showed that compared to day 1 after seeding, the mRNA expression of CYP1A2, 2A19, 2D25, 2E1 were reduced approximately 90 % after day 2 in culture. Following the next 5 days in culture the mRNA expression remained stable at a low level compared to day 1. The mRNA expression of CYP2C33 and 3A29 was not changed by time in culture. These findings are in accordance with the results found by Skaanild and Friis (2000), Loven *et al* (2005) and Gillberg *et al* (2006), that showed that in porcine hepatocytes, the CYP2A dependent activity was reduced up to 80% during the first day in culture. Additionally the study by Skaanild and Friis (2000) showed a concurrently reduction in CYP2A

dependent activity and protein expression. Moreover it has been shown that CYP1A dependent activity decreases with time in culture (Hg *et al.* 1995; Zhou *et al.* 1998), while CYP2E1 protein expression is not changed within 24 hours after seeding (Doran *et al.* 2002). As the present study showed that mRNA expression of CYP1A2 and 2A19 is reduced, it could be suggested that the reduction in CYP dependent activity is caused by a reduction on the transcriptional level. The unchanged expression of CYP3A29 mRNA found in the present study is in accordance with Gillberg *et al* (2006) and Skaanild and Friis (2000), that showed constant protein expression and CYP3A dependent activity for up to 75 hours of culturing. However, other studies have showed both increased (Loven *et al.* 2005) and decreased (Monshouwer *et al.* 1998) CYP3A dependent activity. Taken together, the present findings suggest that the reduction of CYP protein expression and activity found with time in culture is due to reduced mRNA expression. In the present study we showed that for none of the investigated CYPs, there were differences between time dependent CYP mRNA expression in freshly isolated and cryopreserved hepatocytes. This suggests that cryopreserved hepatocytes can be used to study CYP mRNA expression.

To test the ability and the magnitude of which the hepatocytes can regulate CYP mRNA we treated them with standard inducers. β-Naphtoflavone and dexamethasone are commonly used to activate the receptors AhR and PXR, respectively, while CITCO and TCPOBOP are used to activate CAR. However, as supported by the current results, the inducers can activate other receptors either directly or indirectly, via cross-talk or via their metabolites. In the present study, β-naphtoflavone proved to be very specific in inducing an approximately 5 fold increase in CYP1A2 mRNA expression, probably by activating AhR. Porcine CYP1A induction by β-naphtoflavone has been shown before

both *in vivo* (Messina *et al.* 2009; Nannelli *et al.* 2009) and *in vitro* (Monshouwer *et al.* 1998). TCPOBOP has previously, like in the current study, shown to have no effect on CYP2A expression (Gillberg *et al.* 2006) in porcine hepatocytes, in contrast to murine hepatocytes. Moreover, the study by Gillberg *et al* (2006) showed that CITCO induced CYP2A dependent activity. Surprisingly, our study showed no changes in CYP2A19 mRNA in hepatocytes treated with CITCO, while an increase in CYP1A2 mRNA expression was observed. This discrepancy can be explained by the difference in investigated response, mRNA expression vs. enzyme activity. Dexamethasone has previously been shown to increase CYP3A in porcine hepatocytes (Monshouwer *et al.* 1998). In the current study dexamethasone induced CYP3A29 and 1A2 mRNA expression, while the increase in 2C33 almost reached significant levels ($p = 0.054$). This result is supported by studies using human hepatocytes, showing that dexamethasone treatment increased both CYP3A4, CYP2C8 and CYP1A1 (Lai *et al.* 2004; Pascussi *et al.* 2000). This suggests that dexamethasone has a complex mode of action in relation to nuclear receptors and gene regulation. Taken together, the results show that, even though CYP expression is suppressed with time in culture, the hepatocytes are still able to increase CYP mRNA by at least 5 fold.

To investigate the potential of inducing CYPs with bioactive compounds, we treated hepatocytes with a sesquiterpene lactone, artemisinin, and a coumarine derivative, scoparone. Both sesquiterpene lactones and coumarines are found in chicory (Bais and Ravishankar, 2001). Artemisinin increased CYP1A2 and 3A29 mRNA expression by up to 3.5 times, while CYP2C33 and 2D25 was increased up to 2 times. This suggests that artemisinin simultaneously activates AhR, CAR and PXR. This is further supported by a study using a gene reporter assay, which showed that artemisinin activates both

human CAR and PXR, as well as induced CYP3A4 and 2C19 mRNA expression in human hepatocytes (Burk *et al.* 2005). Simonsson *et al* (2006) also found that artemisinin activated the human CAR and induced a weak but reproducible increase in CYP1A2 mRNA in mice treated with artemisinin for 4 days. Scoparone treatment increased the mRNA expression of CYP1A2 and 2D25, suggesting an activation of both AhR and CAR. Previous studies using primary human and mice hepatocytes, have shown that scoparone can induce CYP2B6 and CYP2B10 mRNA expression, by activating CAR (Huang *et al.* 2004; Yang *et al.* 2011).

A number of studies have shown that plant compounds can change CYP expression and activity (Chang 2009; Chang and Waxman 2006). A previous study in our lab investigated the effect of feeding dried chicory root to pigs (Rasmussen *et al.* 2011d). In the group of pigs which had been giving 10 % dried chicory root in their feed for the last 16 days before slaughter, there was up to 9 times increase in CYP1A2 and 2A19 mRNA and a small increase in CYP2E1 mRNA. Correspondingly, there was a subsequent increase in CYP1A2 and 2A19 protein expression and activity. Surprisingly the results in the present study show that CYP mRNA expression is down-regulated in hepatocytes exposed to a methanol extract of chicory root. Of the investigated CYPs significant down-regulation was shown for CYP1A2, 2C33, 2D25 and 3A29, while CYP2A19 and 2E1 was not affected. The decrease in CYP expression was larges in the hepatocytes treated with high concentrations of the chicory extract. The mechanisms behind this down-regulation needs to be investigated in future studies. However, it is worth noticing that the same CYP isoforms are up-regulated in the dexamethasone and artemisinin treated hepatocytes. Taken together, this can suggest that these CYPs might be regulated together and that the chicory extract act as an inverse agonist of these CYP

regulatory pathways. Moreover, the discrepancies between the *in vivo* and *in vitro* effects of chicory need further investigation. When extracting plant compounds for use in *in vitro* models, the possibility of excluding bioactive compound due to solubility problems exists. Another explanation is post absorptive modification of the chicory compounds in the enterocytes in gastrointestinal tract of the animal, before they enter the liver, potentially making them ligands for the CYP regulating receptors. Likewise, more complex events like changes in hormone concentration by chicory can also effect CYP expression. Taken together, the results suggest that compounds in chicory can have very strong and diverse effects on the liver metabolism.

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Authorship contributions

Participated in research design: Krøyer Rasmussen, Ekstrand

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Contributed new reagents or analytic tools: Krøyer Rasmussen, Lindgaard Jensen, Ekstrand

Performed data analysis: Krøyer Rasmussen, Ekstrand

Wrote or contributed to the writing of the manuscript: Krøyer Rasmussen, Ekstrand

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Figure legends

Figure 1

Cytochrome P450 mRNA expression in freshly isolated (closed circles) and cryopreserved (open circles) primary porcine hepatocytes (n = 4). mRNA expression of A) CYP1A2, B) 2A19, C) 2C33, D) 2D25, E) 2E1 and F) 3A29 was determined on day 1 (24 h after seeding), 2, 3, 6 and 7 in culture. Data are mean ± SEM.

Figure 2

Induction of cytochrome P450 mRNA in primary porcine hepatocytes by standard inducers. Hepatocytes (n = 4) were treated with A) 100 µM β-naphtoflavone, B) 100 µM CITCO, C) 50 µM dexamethasone or D) 5 nM TCOPBOP for 24 h. Data are mean ± SEM. * $p < 0.05$ and *** $p < 0.001$ significantly different from control (no inducers). Bars not sharing subscription are different from each other ($p < 0.05$). Significance was evaluated using one-way ANOVA, followed by Tukeys post hoc test

Figure 3

Induction of cytochrome P450 mRNA in primary porcine hepatocytes by 1-100 µM artemisinin and 10-100 µM scoparone. Hepatocytes (n = 4) were treated with A) artemisinin or B) scoparone for 24 h in concentration as indicated in the figure. Data are mean ± SEM. * $p < 0.05$ ** $p < 0.01$ significantly different from control (no artemisinin or scoparone). # significantly difference between concentrations. Significance was evaluated using one-way ANOVA, followed by Tukeys post hoc test.

Figure 4

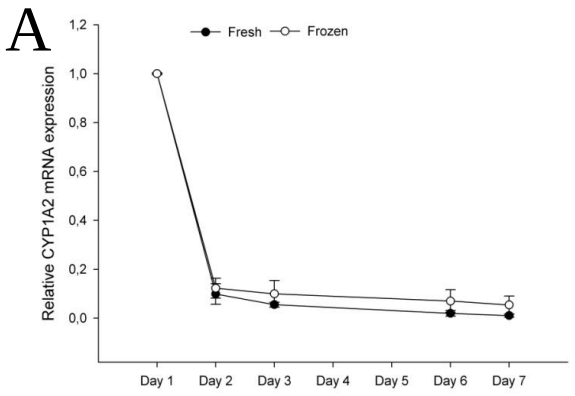
Cytochrome P450 mRNA expression in primary porcine hepatocytes treated with a methanolic extract of chicory root. Hepatocytes (n = 4) were treated with different dilutions of the original extract for 24 hours as indicated in the figure. Data are mean ± SEM. * significantly different from control samples (no chicory extract). Significance was evaluated using Student's t-test with Bonferroni's correction.

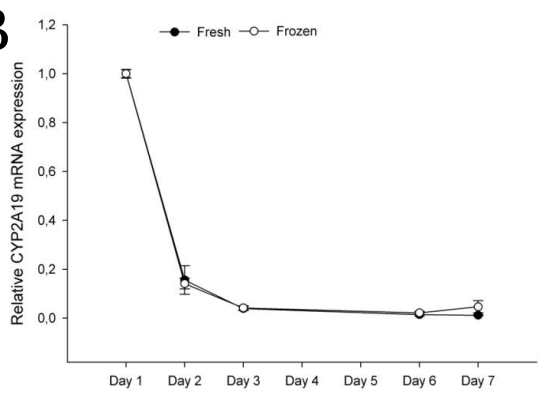
Tables

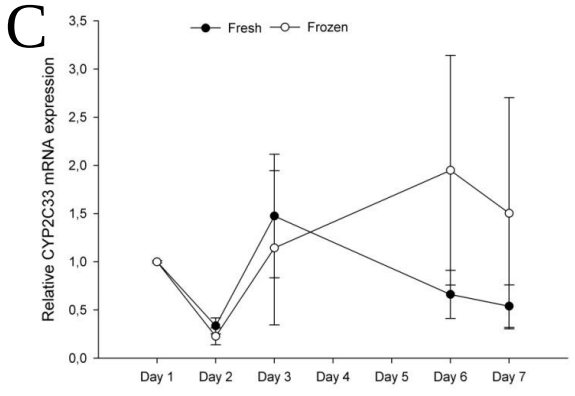
Table 1. Viability of fresh and cryopreserved porcine hepatocytes.

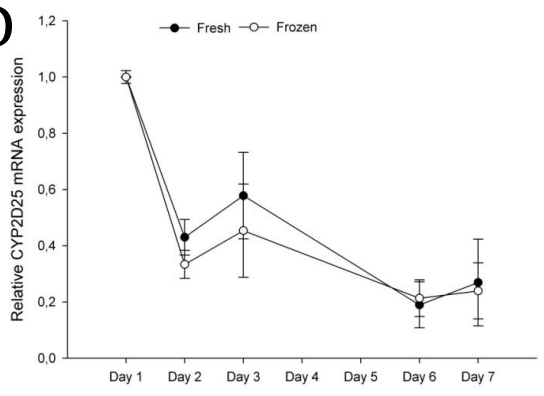
	Viability (%)	
	Fresh	Cryopreserved
Pig A	95	79
Pig B	92	72
Pig C	84	69
Pig D	85	74
Pig E	86	71
Average	*88,4*	*73,0*
SD	*4,8*	*3,8*

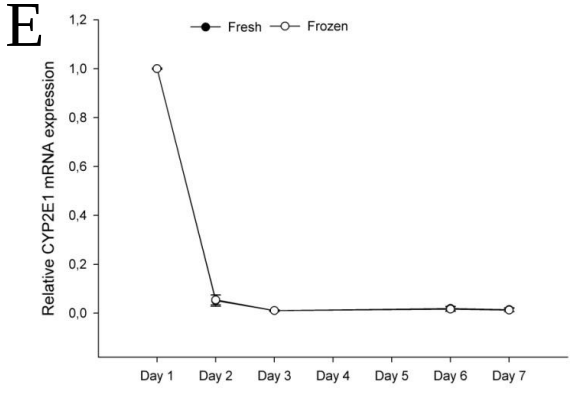
Figure 1


A
Fresh
Frozen
Relative CYP1A2 mRNA expression
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7


B
Fresh
Frozen
Relative CYP2A19 mRNA expression
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7


C
Fresh
Frozen
Relative CYP2C33 mRNA expression
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7


D
Fresh
Frozen
Relative CYP2D25 mRNA expression
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7


E
Fresh
Frozen
Relative CYP2E1 mRNA expression
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7

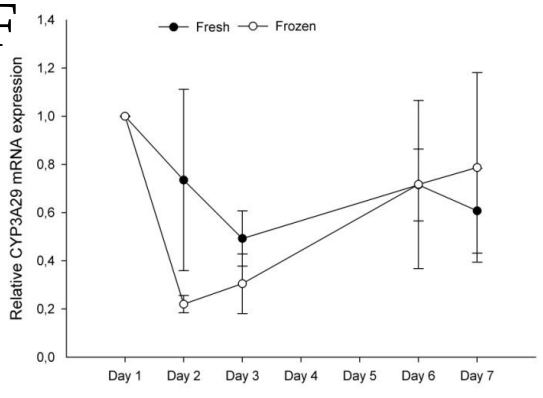

F
Fresh
Frozen
Relative CYP3A29 mRNA expression
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7

Figure 2

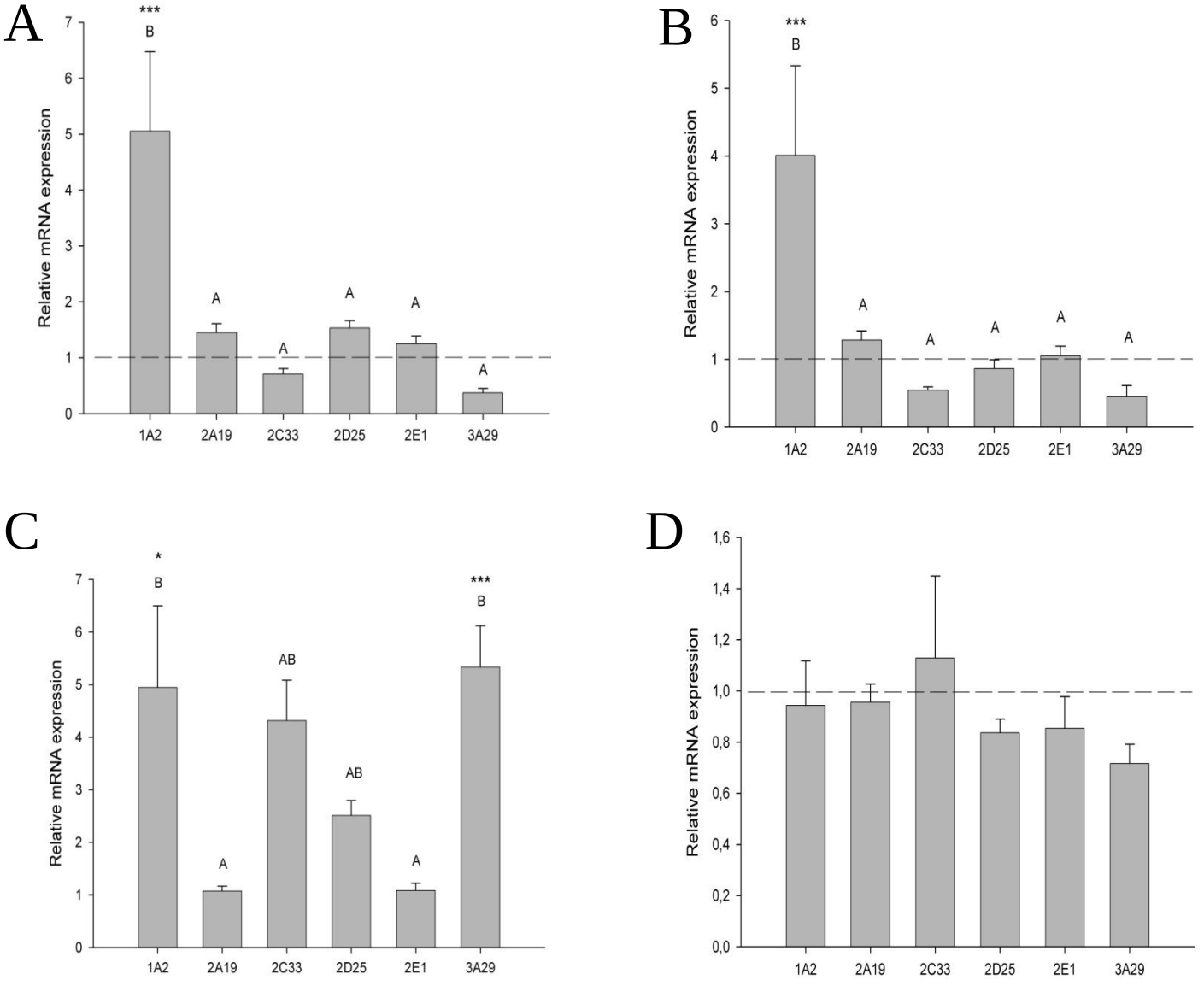

A
B
C
D
Relative mRNA expression
1A2
2A19
2C33
2D25
2E1
3A29
***
*
B
A
AB

Figure 3

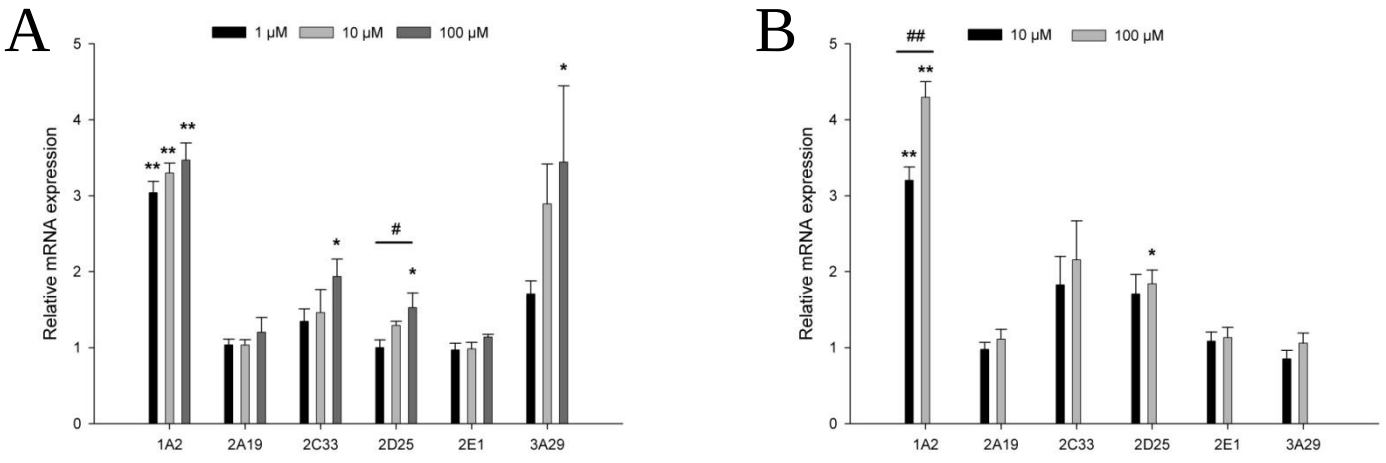
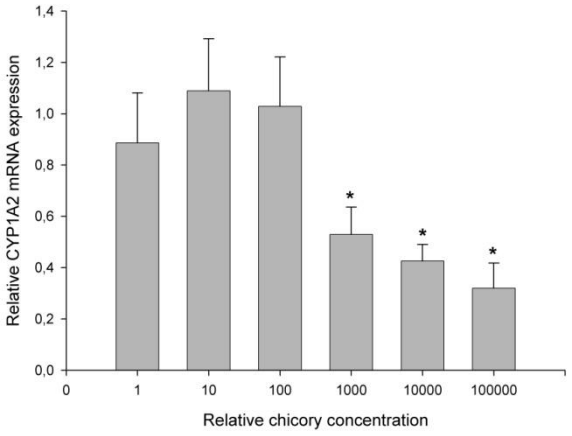

A
1 μM
10 μM
100 μM
Relative mRNA expression
0
1
2
3
4
5
1A2
2A19
2C33
2D25
2E1
3A29
B
##
10 μM
100 μM
Relative mRNA expression
0
1
2
3
4
5
1A2
2A19
2C33
2D25
2E1
3A29

Figure 4

A



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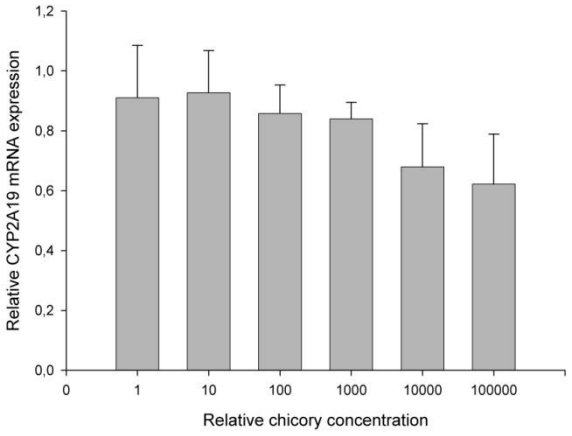

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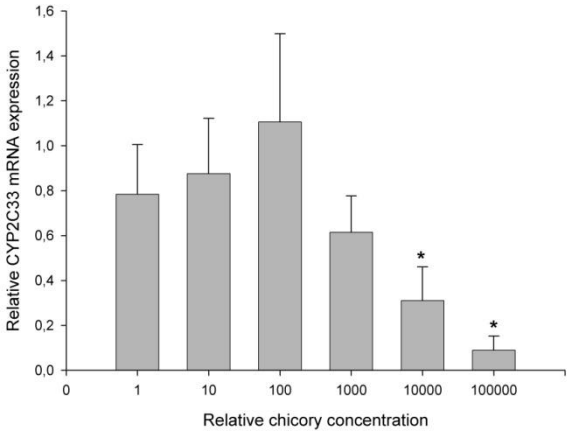

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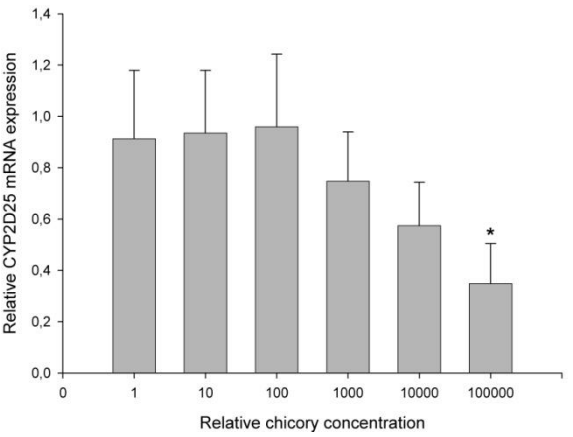
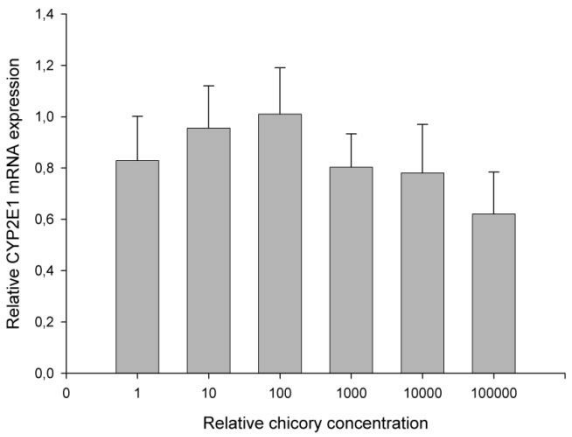


E



F