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The urinary metabolome in female mink (*Mustela neovison*) shows distinct changes in protein and lipid metabolism during the transition from diapause to implantation

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Abstract

Introduction The mink exhibit an obligatory diapause. The metabolic changes during the transition from diapause to implantation and established pregnancy are currently unknown.

Objectives The study aimed to characterize changes in the urinary metabolome in mink during the period from mating to early gestation and to identify the metabolites involved.

Methods Urine samples were collected from 56 female mink on March 24, April 8, and April 15, covering the period from mating to early pregnancy. The urine samples were subjected to non-targeted LC-MS metabolomics. Processed data were evaluated by principal component analysis (PCA) and the peak area of identified metabolites were subjected to ANOVA.

Results The samples showed clear clustering according to sampling date in a PCA scores plot, and 35 metabolites differing significantly between sampling days were identified. The excretion of dicarboxylic acids and acylcarnitines of dicarboxylic acids exhibited a decline on April 8, and the same trend was observed for four unidentified metabolites, two of which were putatively identified as acids of the furan fatty acid type. The decreased excretion of lipid components was suggested to be a result of increased oxidation of these compounds. In contrast, the excretion of amino acid-related metabolites showed an increase on April 8 which was attributed to increased metabolism of amino acids at this time point.

Conclusion

The urinary metabolic profile of mink showed distinct changes during the period studied. The major changes were observed at the time of implantation where increases in the lipid and protein metabolism were evident.

Keywords: Metabolomics, dicarboxylic acids, acylcarnitines, furan fatty acids, bacterial amino acid metabolites

1 Introduction

Mink (*Mustela neovison*) are seasonal breeders regulated by photoperiod, and they usually mate during the month of March (Sundqvist et al. 1989). Mink go into obligate embryonic diapause which is characterized by arrest in embryo development at blastocyst stage (Lopes et al. 2004). The true gestation has a length of 30 ± 3 d in mink (Tauson et al. 1994) but due to the diapause the gestational period in mink may vary from 42 to 75 d and hence the stage in gestation cannot be determined until after birth. The diapause acts to uncouple breeding from parturition to maximize the survival of the offspring (Murphy 2012). The duration of the diapause is under hormonal as well as uterine control (Lopes et al. 2004) but there is still much to learn about the details of the control of embryonic diapause. Embryonic diapause has been identified in over 130 mammalian species (Fenelon et al. 2014), however, the existence of diapause in primates, including humans, is discussed (Renfree 2015; Ptak et al. 2013).

During pregnancy, a substantial regulation of the metabolism of all nutrients takes place in order to ensure optimal growth and development of the fetus (King 2000). In humans, the changes in the metabolism involve alterations in the protein metabolism to ensure e.g. that protein is deposited during the last trimester of the pregnancy (Elango and Ball 2016) and changes in lipid metabolism where fat deposition in early gestation is followed by lipolysis in late gestation (Lindsay et al. 2016).

Mink are strictly carnivorous and they have a high requirement for protein. This requirement varies during the production cycle, but the knowledge of the actual demand at all parts of the production cycle is at present insufficient. The importance of gestational nutrition is well accepted and this is especially true for protein provision (Matthiesen et al. 2016).

Metabolomics, the measurement of all small molecules, metabolites, present in a sample of interest, e.g. a urine or a blood sample, is ideally suited for studies of metabolic changes during pregnancy and is being increasingly used in studies regarding human pregnancy (Luan et al. 2014; Pinto et al. 2015; Lindsay et al. 2016). In healthy humans, Diaz and coworkers showed that urine samples collected during the first, second, and third trimester of pregnancy displayed a trajectory across pregnancy (Diaz et al. 2013). Pregnancy-

induced changes were also obvious in the plasma metabolome (Luan et al. 2014). Apart from studies in healthy human pregnancies, metabolomics has been used to characterize and find biomarkers for a range of relevant prenatal diseases (Huynh et al. 2014; Graca et al. 2012; Kenny et al. 2010).

The aim of the present study was to investigate the metabolic changes reflected in the urinary metabolome in mink in the period from mating to established pregnancy. It has, to my knowledge, never been studied how the urinary metabolome changes in species with embryonic diapause during the period from mating to established pregnancy and whether these changes differ from those observed in humans pregnancies. I hypothesized that urine samples would cluster according to sampling day like it has been observed for human subjects and that it would be possible to identify metabolites and hence metabolic pathways changing during early pregnancy indicating metabolic adaptation to the altered physiological state of the mink dam.

2 Materials and Methods

2.1 Animals, diets, and housing

The experiment was performed on a commercial farm (Lund Mink, Sunds, Denmark) in the period from March 24 to April 15, 2015. The study comprised 56 one-year-old female mink (*Mustela neovison*), 34 of the color type brown and 22 of the color type pearl. A total of 96 urine samples were collected, hence the number of urine samples collected per female ranged between one and three.

The animals were housed individually in standard mink cages (L: 90 cm x W: 30 cm H: x 45 cm). All cages had access to a wooden nest box (L: 28 cm x W: 30 cm x H: 24 cm) embedded and covered with straw. In addition, each cage was equipped with a shelf – one wire tube cylinder (l: 32 cm, diameter: 11 cm) fixed to the cage ceiling – according to the Danish legislation (Ministry of Food, Agriculture and Fisheries of Denmark, 2006). The cages were raised above the ground in an unheated shed at the mink farm.

The animals were fed a conventional wet diet for the winter and gestational period from a local feed kitchen (Supplementary Table 1). The animals were fed once a day between 11 a.m. and 12 a.m.

All female mink were mated twice with the same male according to standard farm procedures for the first time between March 4 and 13 and for the second time eight or nine days later. The mink were exposed to natural lighting.

During the period from April 24 to May 5 all cage units were checked once daily to register time of litter birth (Day 0) and to collect dead kits. The total number of born kits is defined as all kits delivered (being alive or dead), and mortality is the proportion between the number of dead kits and the total number born in that litter. The kits were only counted on Day 0. Three brown mink and two mink of the color type pearl were barren, i.e. with no signs of kits delivered. These barren females were excluded from the data analysis, reducing the number of experimental mink to 51 dams and the number of urine samples to 84.

2.2 Collection of urine samples

Urine samples were collected on March 24, April 8, and April 15, 2015. The times were selected to cover the period from mating to early pregnancy. Samples were collected by catching the female and holding her over the edge of the cage. Most mink spontaneously urinate during this procedure and the urine was collected in a plastic cup. The collected urine was divided into test tubes and immediately frozen. The samples were stored at -80°C until analysis.

2.3 Solvents and standards

High-performance liquid chromatography (HPLC)-grade acetonitrile was purchased from VWR (West Chester, PA, USA) and formic acid from Fluka (Fluka, Sigma-Aldrich, St. Louis, MO, USA). Glycocholic acid

(Glycine- ^{13}C) and 4-Chloro-DL-phenylalanine purchased from Sigma (Sigma, MO, USA) were used as internal standards.

The following standards were used for identifications: acetylphenylalanine, ascorbic acid, azelaic acid, betaine, carnitine, cinnamoylglycine, citric acid, creatine, creatinine, 4-guanidinobutanoic acid, hippuric acid, 2-hydroxyisocaproic acid, indoxyl sulfate, kynurenic acid, 3-methylhistidine, pantothenic acid, pimelic acid, 2-piperidinone, riboflavin, sebacic acid, suberic acid, taurine, uric acid, and xanthurenic acid and were all purchased from Sigma (Sigma, MO, USA). The standards for N-methyl-2-pyridoxone-5-carboxamide and phenylacetylglycine were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). A p-cresol sulfate standard was purchased from Alsachim (Illkirch-Graffenstaden, France) and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) was obtained from Cayman Chemical (Ann Arbor, MI, USA).

2.4 Non-targeted LC-MS metabolomics analysis of urine

The urine samples (90 μl) were diluted with water (90 μl) and 20 μl of internal standard (Glycocholic acid (Glycine- ^{13}C) and p-chlorophenylalanine, final concentration 0.01 mg/ml) was added. The samples were left at 4°C for 20 min for protein precipitation. The samples were centrifuged (17950 x g, 10 min, 4°C) and the supernatants were transferred to vials with a micro insert. The sample injection volume was 2 μl .

Chromatographic separation was performed on a Dionex UltiMate 3000 RSLC Binary UHPLC System (Thermo Scientific Dionex, Sunnyvale, CA) equipped with an HSS T3 C18 UHPLC column, 1.8 μm , 100 x 2.1 mm (Waters Corporation, Milford, MA). The column was maintained at 30°C. The mobile phases were 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 0.4 ml/min. The gradient program was as follows: 0-12 min, linear gradient from 5 to 90% B; 12-12.3 min, 90% B and return to initial conditions in 0.2 min. Corresponding changes in A were made. The column was re-equilibrated at 5% B for 2 min in the beginning of each run.

The eluent was introduced into an Ultra-High Resolution Qq-Time-Of-Flight mass spectrometer (Impact HD, Bruker Daltonics GmbH, Bremen, Germany) by electrospray ionization with the capillary set in the positive and negative mode to 4500 V and 3600 V, respectively. End plate offset voltage was set to 500 V, the dry gas flow and temperature were 8 l/min and 200°C, respectively, and nebulizer pressure was set to 1.8 bar. The scan range was from 50 to 1000 m/z at a sampling rate of 1 Hz. The collision energy during the MS scan was set to 6 eV. Lithium formate at a concentration of 5 mM in water-isopropanol-formic acid (50:50:0.2, v/v) was employed as an external calibrant in the beginning of each chromatographic and run with an independent syringe pump. For MS/MS analysis, Ar was used as the collision gas and collisions were carried out at energies from 10-40 EV. All other parameters were the same as above.

As quality controls, a blank sample (5% acetonitrile) and a pooled urine sample (quality control, QC) were injected after each six samples to evaluate the analytical system performance by potential cross-contamination from samples, loss of sensitivity, and system reproducibility during the run.

2.5 Data processing and metabolite identification

Acquired mass spectra were calibrated and peak detection was performed using the “Find Molecular Features” option in Compass DataAnalysis Version 4.2 (Bruker Daltonics GmbH). The spectra were exported to Bruker Compass ProfileAnalysis 2.1 for initial statistical evaluation. A matrix was generated with retention time, m/z and respective intensities. This matrix was exported to LatentIX 2.10 (Latent5 Aps.). The data were normalized according to the peak area of the internal standard to compensate for variability in sample processing and analytical platform operation. Prior to principal component analysis (PCA) data were Pareto-scaled. Pareto scaling reduces the relative importance of large values but keeps the data structure partially intact (van den Berg et al. 2006). In negative mode, one outlier was removed based on 95% CI and plots of residual variance versus Hotelling’s T^2 after which the model was recalculated. In order to detect metabolite ions with the greatest influence on clustering partial least squares-discriminant analysis (PLS-

DA) models for pair-wise comparison of the sampling dates were constructed. The models were constructed using a calibration dataset consisting of two thirds of the samples with the principal variables accounting for 96-98 % of the variation and validation was performed with a validation dataset consisting of the last third of the samples. The predictive value of the models was evaluated and plots of scaled regression coefficients, which show the contribution of each variable to the score formation, were used to select metabolites for identification. Compounds were identified based on queries in the METLIN (<http://metlin.scripps.edu/>), Human Metabolome Database (<http://www.hmdb.ca/>), and LIPID MAPS (<http://www.lipidmaps.org/>) online databases for obtaining possible chemical structures using accurate mass and mass spectrometric fragmentation patterns. The identification of the annotated compounds was confirmed with standards, when available, on the same analytical system under the same conditions (validation based on retention time and mass spectra). For metabolites where no suggestions were found in the databases the utility "Smart formula" in Compass DataAnalysis Version 4.2 was used to suggest molecular formulas of the metabolites. The level of metabolite identification (Sumner et al. 2007) is indicated in Table 1.

2.6 Statistical analysis

Analysis of variance was carried out on variables selected from PCA with date, color, and the interaction between date and color as sources of variation using the GLM procedure of SAS release 9.2. Tests were made at the 5% significance level. Least squares means were compared using Fisher's least-significance-difference procedure (Milliken and Johnson 1984). Results are presented as least squares means (lsmeans) with their SEM. However, to obtain normality data some of the metabolites had to be analyzed on a logarithmic scale. As confidence intervals on the original scale are not symmetric around the parameter estimates, the confidence limits rather than the standard errors are presented for these metabolites. The lsmeans, SEM, and confidence limits are all normalized to the intensity of the most abundant metabolite in

the chromatograms obtained in positive mode (Fig. 1). The relative peak area of this metabolite on March 24 is set to 100 and the peak areas of the other metabolites were calculated according to this. To correct for multiple comparisons, false discovery rate q values were calculated (Benjamini and Hochberg 1995) with a significance threshold at $q < 0.46$. The fold-change in metabolite peak area was calculated as the relation between the peak area on April 8 and March 24 and the peak area on April 15 and March 24.

3 Results

3.1 Reproduction and kit mortality

The mink gave birth from April 27 to May 6 and based on a mean duration of gestation of 30 d implantation was estimated to have taken place between March 28 and April 6. Hence, the first urine sample was collected when the embryos were still in the embryonic diapause and when the samples were collected on April 8, implantation had taken place in all mink (Fig. 2).

The number of barren females was two and three for brown and pearl mink, respectively (Supplementary Table 2). Due to the low number of barren females, this was not statistically evaluated. The number of kits per litter did not differ between color type ($p = 0.70$). It ranged from 6.61 ± 0.46 in brown mink to 6.90 ± 0.57 in mink of the color type pearl. The number of stillborn kits was almost twice as high in mink of the color type pearl as in brown mink ($p = 0.03$).

3.2 Multivariate data analysis

The blank samples showed no addition of peaks indicating that no cross-contamination between the samples occurred (results not shown). Furthermore, the chromatograms of the reinjected QC sample were indistinguishable which was confirmed by a close clustering of the QC samples in the PCA scores plot (results not shown), verifying the stability and reproducibility of the analytical system.

After preprocessing, the number of metabolites (variables) in positive and negative mode was 774 and 835, respectively. The PCA scores plot of the urine samples analyzed in positive and negative ionization mode

are shown in Fig. 3. In positive ionization mode (Fig. 3a) a clear separation between sampling days is observed. Samples from March 24 are more scattered than samples from April 8 and 15 indicating a higher variation between the samples from March 24. In negative ionization mode (Fig. 3b) the samples from March 24 are clearly separated from the other sampling days whereas samples from April 8 and 15 are somewhat overlapping. The amount of variability accounted for by the first two principal components was 40.39% and 38.70% in positive and negative mode, respectively. No clustering according to the color type of the mink was observed (results not shown). PLS-DA plots and their corresponding regression coefficients plots for pair-wise comparison of the sampling days for selection of metabolites for identification are shown in supplementary Fig. 1. The figures show a high predictive value for all models, no misclassification of the validation datasets was observed. Metabolites were selected for identification based on the regression coefficient plots and the identified metabolites are marked in supplementary Fig. 1.

3.3 Metabolites in urine

Metabolites responsible for the separation of the sampling days are shown in Table 1 and the fold-change of the identified metabolic features is shown in Fig. 4 where the blue color indicates a fold-change below one, and the red color indicates a fold-change above one.

The excretion of a group of dicarboxylic acids (pimelic, suberic, azelaic, and sebacic acid) decreased from March 24 to April 8 and increased again on April 15 (Table 1 and Supplementary Fig. 2). A similar trend was observed for four metabolites, which were not possible to identify based on submitting the masses to the METLIN database or the Human Metabolome Database. One of these metabolites ($[M+H]^+$ 231.076/5.41) was the most abundant peak in the chromatogram in positive mode (Fig. 1a), whereas the ion $[M-H]^-$ 239.092/5.80 was the most abundant peak in the chromatogram in negative mode (Fig. 1b). The fragmentation patterns of the unidentified metabolites are shown in Supplementary Fig. 3. The fragmentation of the ion $[M+H]^+$ 231.076/5.41 showed the loss of 46.0558 amu in positive mode and 43.9857 amu in negative mode. This corresponds to loss of HCOOH and CO₂, respectively, indicating that

the ion contains an acid group. Furthermore, two times loss of 18.0107, H₂O, indicated the presence of acid or alcohol groups. Use of “Smart formula” indicated that the molecular formula of the ion was C₁₂H₁₀N₂O₃, which corresponds to the measured mass with a mass error of 8.2 ppm. Search for the ion [M+H]⁺ 241.107/5.80 suggested that it could be 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), and the fragmentation pattern supported this. However, running the standard showed that this identification was not correct (Supplementary Fig. 4). Nevertheless, because the fragmentation pattern showed loss of two acid groups and the mass accuracy (2.49 ppm) between the measured mass and the theoretical mass, it is suggested that the metabolite is an isomer of CMPF. The fragmentation pattern of the ion [M+H]⁺ 269.138/6.84 showed that the molecule contained two acid groups and the suggested formula was C₁₄H₂₀O₅ – hence this metabolite was suggested to have structural similarities to the CMPF-isomer. A tentative identification of the ion [M+H]⁺ 268.154/7.39 was not possible. In positive mode both a Na- and a K-adduct were seen (results not shown) indicating that the metabolite is an acid; furthermore, the elution of the metabolite late in the chromatogram supports this. However, the fragmentation patterns did not provide any further information and the metabolite remained unidentified.

Carnitine excretion was low on March 24. It increased significantly on April 8 and was medium on April 15. A range of acylcarnitines were identified based on the characteristic MS/MS fragmentation pattern with the loss of 85 amu (Kivilompolo et al. 2013). The acylcarnitines were all tentatively identified as carnitines of dicarboxylic acids based on their possible formula. The excretion of the acylcarnitines was generally lowest on April 8 (Supplementary Fig. 2).

The excretion of betaine was highest prior to implantation and the excretion declined during the subsequent samplings. Ascorbic acid displayed a doubled excretion from March 24 to the collections in April. N-Methyl-2-pyridone-5-carboxamide (2PY) is a metabolite of nicotinamide-adenine dinucleotide that is a product of vitamin B3, and pantothenic acid and riboflavin are members of the vitamin B complex as well. The excretion of 2PY and pantothenic acid is high prior to implantation, whereas the excretion of riboflavin follows a different pattern being highest right after implantation.

Urinary excretion of creatinine decreased from March 24 to April 8 and remained constant until April 15. The level of creatine and 3-methylhistidine was significantly higher in the urine samples from April 8 than from March 24 and April 15. Cinnamoylglycine, p-cresol sulfate, indoxyl sulfate, phenylacetylglycine, hippuric acid, and 2-hydroxyisocaproic acid are metabolites produced by the gut microflora. The excretion of these metabolites was highest on April 8. A low excretion was observed on March 24 while the excretion on April 15 was medium. 4-Guanidinobutanoic acid, acetylphenylalanine, xanthurenic acid, and kynurenic acid are metabolites from the metabolism of arginine and proline, phenylalanine, and tryptophan, respectively. The excretion of these compounds was highest on April 8 whereas the excretion on March 24 and April 15 was comparable. Taurine, 2-piperidinone and uric acid were found in low concentrations on March 24. The concentrations increased on April 8 and a decrease was observed on April 15, but the concentration was, however, still higher than on March 24. Further, the excretion of citric acid was higher on April 15 than on March 24 and April 8.

Overall, there was a very clear difference between metabolites related to fat metabolism and metabolites related to protein metabolism. The former metabolites displayed a fold-change below one from March 24 to April 8 whereas the latter show a fold-change larger than one between April 8 and March 24 (Fig. 4).

4 Discussion

The present investigation is to my knowledge the first to show the distinct changes to the urinary metabolome during early pregnancy in a species with obligate embryonic diapause.

The multivariate data analysis of the mink urine metabolome showed a clear discrimination between the sampling dates, however, a relatively low amount of variability was contained in the first two PC's. This is an indication that factors other than the sampling date are important for the variability of the metabolome. The larger variation between the samples collected on March 24 compared to samples collected on April 8 and 15 furthermore suggests that the individual variation between female mink was reduced after implantation likely due to metabolism being focused on supplying the fetuses at this time. The amount of

variability contained in the first two PCs was comparable to that observed in a human study (Diaz et al. 2013) where urine samples were collected during the first, second and third trimester.

The mink excreted dicarboxylic acids during the entire experimental period. Excretion of dicarboxylic acids has not been reported in pregnant women, and urinary excretion of dicarboxylic acids is in non-pregnant humans characterized as a metabolic disorder – dicarboxylic aciduria (Tserng et al. 1996) which indicates an increased fatty acid mobilization or a defect in fatty acid mitochondrial β -oxidation (Tserng et al. 1991).

Dicarboxylic acids are derived either by β -oxidation of longer-chain dicarboxylic acids or by ω -oxidation in the microsomal membranes (Mingrone and Castagneto 2006). Dicarboxylic acids are β -oxidized in the peroxisomes and mitochondria (Mingrone et al. 2013). The pathways for transportation of dicarboxylic acids to the mitochondria do not require the carnitine shuttle (Mingrone et al. 2013), but carnitine consuming transportation has been shown for sebacic and dodecanedioic acid as well (Kølvraa and Gregersen 1986). In the present study, a number of dicarboxyl-carnitines (C_5 - C_{12}) were tentatively identified indicating that the carnitine shuttle exists for shorter dicarboxylic acids in mink. The excretion of dicarboxylic acids and their carnitines was lowest on April 8 indicating a higher oxidation of these compounds at the time of implantation. However, the excretion of free carnitine followed the opposite pattern being highest on April 8. This corresponds to humans where the concentration of free carnitine has been shown to be higher in pregnant than in non-pregnant women, both in urine and plasma, and the concentration was decreasing during each trimester (Cho and Cha 2005; Luan et al. 2014). In pregnant women, the excretion of acylcarnitines declined during pregnancy (Cho and Cha 2005). In plasma, total acylcarnitines did not change during pregnancy (Luan et al. 2014), but looking at the individual acylcarnitines differences related to the chain length were seen (Luan et al. 2014; Lindsay et al. 2016). The level of short and medium-chain acylcarnitines decreased during pregnancy which is not in accordance with the increased excretion observed from April 8 to 15 in the present study, but the correlation between the concentration of acylcarnitines in plasma and urine is not known.

The tentative identification of a CMPF-isomer and a second acid of similar structure may be biomarkers of the high intake of fish in mink. The furan fatty acid, CMPF, has in several human studies been shown to be elevated in human subjects consuming fish or fish oil (Hanhineva et al. 2015; Zheng et al. 2016). The decreased excretion of these compounds during the second sampling was indicative of higher oxidation of these compounds during this period.

The excretion of betaine declined during implantation. Betaine may be derived from the diet or synthesized by irreversible conversion of choline to betaine (Craig 2004), and the decline in betaine excretion in the present study may be due to an increased demand for choline as well as betaine. Betaine is a methyl donor for the remethylation of homocysteine to methionine (Ueland et al. 2005), the supply of methyl groups is vital throughout pregnancy, and it is suggested to have an important function during early development of the fetus (Lever and Slow 2010).

The excretion of three water-soluble vitamins, ascorbic acid, pantothenic acid, and riboflavin, and a metabolite of niacin differed between the collection days. It has previously been reported in rats and humans that the excretion of water-soluble vitamins closely reflects the surplus of these vitamins in the body (K. Shibata and Fukuwatari 2013). Urinary excretion of ascorbic acid increased at the time of implantation and remained high. The intake of ascorbic acid did not differ between sampling days, and hence the increased excretion suggests a lower requirement for ascorbic acid during pregnancy in mink. In humans, it was found that pregnancy did not affect the excretion levels of ascorbic acid (K. Shibata et al. 2013). The decrease observed in the excretion of N-Methyl-2-pyridone-5-carboxamide (2PY), a metabolite of niacin, and pantothenic acid implies an increased necessity for niacin and pantothenic acid during pregnancy. In accordance with this, an increased requirement for pantothenic acid during pregnancy was suggested in humans (K. Shibata et al. 2013). The excretion of riboflavin doubled at the time of implantation, which is in contrast to observations in humans where pregnancy did not affect the riboflavin excretion. We have previously shown that mink are fed some B-vitamins in excess (Hedemann et al. 2016).

and the urinary excretion observed in the present study shows that they are fed adequate amounts of the B-vitamins during pregnancy.

Creatine, 3-methylhistidine, and uric acid are all markers of meat intake and/or muscle protein breakdown. The excretion of these metabolites increases at the time of implantation (April 8) which may indicate an increased mobilization of muscle protein (Long et al. 1981) as well as breakdown of animal protein from the diet (Maiuolo et al. 2016; Dragsted 2010). In humans, a decline in plasma concentration of creatine was observed from the non-pregnant state to the first trimester (Pinto et al. 2015). The increased glomerular filtration rate during pregnancy (Cheung and Lafayette 2013) caused a higher excretion of creatine as observed in this study, which is accompanied by a declined excretion of creatinine, the breakdown product of creatine, from the first to the second collection of urine which is in accordance with human observations (Diaz et al. 2013).

The metabolites, cinnamoylglycine, p-cresol sulfate, indoxyl sulfate, hippuric acid, and phenylacetylglycine are bacterial breakdown products of amino acids that have been further metabolized in the liver to facilitate the excretion of these compounds (Wikoff et al. 2009). The increased excretion of these compounds at the time of implantation implies an augmented breakdown by the microbiota or an increased metabolism in the liver. In humans, the composition of the microbiota changed dramatically during pregnancy (Koren et al. 2012) and the increase in Proteobacteria and Actinobacteria, which has been shown to be involved in amino acid metabolism (Wikoff et al. 2009), suggests that the microflora may be involved in the altered excretion of amino acid metabolites. However, the knowledge of the composition of the microflora in mink is scarce and whether remodeling of the gut microbiome takes place during pregnancy in mink remains unknown. 2-hydroxyisocaproic acid is a metabolite of leucine produced by *Clostridium difficile* (Kim et al. 2006). It has previously been identified in humans with short bowel syndrome (Haan et al. 1985). In mink, the bowel is short but whether 2-hydroxyisocaproic acid is a naturally occurring metabolite in mink urine needs to be further studied.

Xanthurenic acid, 4-guanidinobutanoic acid, and acetylphenylalanine are metabolites of the amino acid metabolism. The increased excretion of these metabolites at implantation indicates an increased metabolism that is reduced during the following week. In rats, the excretion of xanthurenic acid increased with the progress in pregnancy (K. Shibata et al. 2003), whereas the excretion levelled out after the increase at implantation in the present study. The present results are in accordance with Matthiesen et al. (2016) who suggested that protein requirement increased in early April around the time of implantation. The excretion of taurine increased at implantation but decreased thereafter. Taurine is critical for fetal growth and reduced taurine excretion has been demonstrated in humans, too, (Diaz et al. 2013) and it is paralleled by decreased plasma taurine concentration (Lindsay et al. 2016).

When comparing the metabolites identified in the current study with metabolites identified in metabolomics studies of healthy human pregnancies, the agreements are few. Mink are strict carnivores whereas humans are omnivores which causes differences in the urinary metabolome due to dietary differences (Scalbert et al. 2014). Furthermore, the analytical platforms used differ. The majority of human studies were analyzed using nuclear magnetic resonance (NMR) (Pinto et al. 2015; Luan et al. 2014; Diaz et al. 2013), whereas the samples in the present study were analyzed using LC-MS. NMR detects metabolites at micro molar or greater concentrations, whereas LC-MS is more sensitive with the ability to measure in the Nano molar range (Lowe and Karban 2014), and this results in different metabolites being identified. However, the present study and the human studies agree that major metabolic changes occur in the amino acid metabolism and, interestingly, the metabolism of acylcarnitines even though they are carnitines of different fat classes in the two species, carboxylic acids in humans (Lindsay et al. 2016) and dicarboxylic acids in mink. However, the major difference between the events during early pregnancy in female mink and humans is that mink are diapausing whereas the existence of diapause in humans is controversial (Renfree 2015) and the discrepancies this may introduce to the urinary metabolome is presently unknown.

5 Conclusions

The present study showed that the metabolic changes taking place in the female mink during implantation and early pregnancy were clearly reflected in the urinary metabolome. The results suggested that the time around implantation is metabolically challenging and that maternal recognition of pregnancy in mink includes a metabolic response. The metabolic changes were, like in humans, primarily associated with the lipid and the amino acid metabolism. Future studies should disclose the possibility to use this knowledge to optimize feeding of female mink during diapause and early pregnancy as well as screening for pregnancy-related health problems.

Compliance with ethical standards

Conflict of interest Mette Skou Hedemann declares no conflicts of interest

Ethical approval:

All applicable international, national, and institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by the author.

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Figure captions:

Fig. 1 Representative base peak chromatograms of a mink urine sample using LC/ESI-QTOFMS in a) positive ionization mode and b) negative ionization mode. The blue dotted lines indicate the time span where the external calibrant is introduced (see Materials and Methods). Four abundant unidentified peaks (UI_1-4) are indicated, the relative peak area of the peaks is shown in Table 1.

Fig. 2 Timeline of the experiment with female mink where urine samples were collected on March 24, April 8 and April 15 (marked with arrows). Mating and birth are marked with black boxes as these are known events. Implantation is marked with a grey box as this is estimated on basis of the dates of birth with an anticipated duration of gestation of 30 days.

Fig. 3 PCA scores plot showing clustering of the female mink urine metabolome by sampling day (March 24, April 8, and April 15) using an unsupervised method in a) positive ionization mode and b) negative ionization mode. The variances accounted for by the principle components are shown on the axes.

Fig. 4 Heatmap alignment of the metabolite fold-change between March 24 and April 8 and between March 24 and April 15 in urine samples collected from female mink on March 24, April 8, and April 15. Names of the identified metabolites and the metabolite groups are listed. The color coding scale indicates the fold changes relative to March 24: blue, fold change < 1; white, fold change =1; red, fold change >1.