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12th Day of the Doctoral Student

Abstracts

19 May 2011
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The 12th Day of the Doctoral Student was held at May 19th, 2011 in the Congress Centre of the FBN. This book contains the abstracts of the presentations given by the advanced doctoral students who have been working on their dissertation for more than one year.

Leibniz-Institut für Nutztierbiologie

Wilhelm-Stahl-Allee 2
18196 Dummerstorf

Tel.: +49 38208 68-5

Fax: +49 38208 68-602

E-Mail: fbn@fbn-dummerstorf.de

Internet: <http://www.fbn-dummerstorf.de>

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“The youth knows mostly every thing,
except one thing:

That the old people also once knew every thing, until they really knew something.”

(Ernest Hemingway)

The Leibniz Institute for Farm Animal Biology is attaching special importance to the promotion of the new generation of academics. With currently 67 PhD students the number of students working in the FBN increased about three fold during the last year.

‘Promotion by demanding’ is the motto of the FBN in supervising the PhD students. The “Day of Doctoral students”, which we are holding annually since 2000, has meanwhile proved as a valuable exhibition-competition of our young academics. Within two groups (“Beginners”, and “Advanced students”) selected PhD projects are presented in a short form. The presentations are evaluated by a jury consisting of the heads of the research units and lecturers present and the best presentation of each group will be awarded. This slightly formalized presentation gives the up-and-coming academics the opportunity to practise an exactly terminated structured scientific talk. These presentations serve to preselect FBN-contributions for participation in important scientific events, such as the annual meetings of the Societies for Animal Breeding (DGfZ/GfT), Reproduction medicine (DVG/DGRM) and Nutrition Physiology (GfE).

Apart from finishing the doctoral thesis in time, contemporary publication of their results is required from the young scientists. With the current issue of the journal “Schriftenreihe des FBN” we give the advanced students the opportunity to publish their results in a citable journal and acquire first experiences in publication of scientific results.

I wish success to all PhD students in their further career as well as personal luck and well-being. As scientists in the area of living sciences, please feel always obliged to respect life, show consideration for the performance of other people, and stay curious to know.

Yours



Prof. Dr. Manfred Schwerin
Director

Transcriptome profiling of bovine endometrial biopsies based on pregnancy succes after transfer of *in vitro* produced embryos

Klara Müller¹ and Siriluck Ponsuksili¹

¹Leibniz Institute for Farm Animal Biology (FBN), FG Functional Genome Analysis

1 Introduction

In the last decades, the main objective in dairy cattle breeding has been on increasing the milk yield per cow. The successful implementation of this onesided focus has resulted in the downgrading of other functional areas, e.g. immune system performance and fertility. Negative correlations between the amplified milk yield and fertility traits have been described [03, 04] and it has been noted that the chances of keeping fertility on an acceptable level via herd management improvements are small [02]. However, since the heritability of characteristics used to evaluate potential fertility is estimated to be relatively low [05], their use as breeding indicators is limited.

The Holstein Friesian is the predominant dairy cattle breed in Germany. Numbers show that reproductive efficiency is significantly higher in heifers than in cows and that the fertility of the latter has dropped much more severely [06], implicating that the effects of husbandry conditions and previous pregnancies overshadow the genetic causes of reproductive potential at this stage.

The challenge in establishing a successful pregnancy in cattle doesn't seem to be fertilisation itself, since observed fertilisation rates have been constantly high (80 – 90%), but the embryo loss which amounts to 30 – 60% (depending e.g. on breed, environmental influences and metabolic status) [07]. The majority of these losses occur during the early stages of pregnancy, between day 8 and 16 after insemination, the phase in which the embryo nidates into the maternal placenta. Taking this into account, the objective of this work is to uncover disparities in the gene expression of differently fertile groups of Holstein Friesian heifers in order to find key genes utilisable as indicators for the level of fertility in high-producing dairy cattle.

2 Materials and methods

Animals: Endometrial samples of 28 Holstein-Friesian heifers were taken with cytobrushes on day 3 and day 7 post oestrus, respectively. The brushes were immersed in RNAlater RNA Stabilization Reagent (QIAGEN) and stored at -80°C. During the next cycle, one *in vitro*-produced embryo each was transplanted into the heifers on day 7 post oestrus. Subsequently,

the animals were monitored for their pregnancy status and the previously taken tissue samples grouped according to pregnancy success.

RNA isolation: Total RNA was isolated by Tri-Reagent (Sigma-Aldrich, D)-phenol-chloroform extraction followed by the purification via the RNeasy Mini Kit and the RNase-free DNase set (both QIAGEN). The RNA was stored at -80°C and used both for microarray analysis and qRT-PCR.

Microarray analysis: The RNA samples were clustered into 3 pools per pregnancy group and timepoint. 500 ng Biotin-labeled cRNA per pool were synthesized using the Gene Chip 3 Express Kit (Affymetrix, USA), then fragmented and hybridized onto Affymetrix GeneChip bovine Genome Arrays. Staining, washing and scanning of the chips were done according to the protocols of the manufacturer.

Data analysis: The expression data were analysed in a first step with Affymetrix® Expression Console™ software, using the MAS5 algorithm to filter out absent probe sets and the PLIER algorithm to normalize the expression values. Then all probe sets with a standard deviation <0.2 were filtered out and the remaining data were used for a mixed model analysis (using pregnancy status and day of cycle as effects) in JMP® Genomics 4.1. The significance level was set at $p < 0.05$.

Pathway analysis: We used Ingenuity® Pathway Analysis (IPA) by Ingenuity® Systems to cluster the significantly regulated probe sets into biological categories and detect regulated pathways between the groups.

Quantitative real time RT-PCR: 10 genes (PTGES, CBR1, HPGD, DNMT3A, ESRRA, DDX46, ASMT, PI15, AP2S1, ZNF529) were chosen for the validation of the microarray results via qRT-PCR. 500ng of total RNA per sample were transcribed into cDNA using the SuperScript™ II Reverse Transcriptase kit (Invitrogen, Germany) with random primers and oligo d(T) primers. The qRT-PCR reactions comprised initial denaturation at 95°C for 10min, 40 cycles of denaturation at 95°C for 15s, annealing at 60°C for 10s and extension/fluorescence acquisition at a primer pair-dependent, individually optimized temperature for 15s and were run on the Roche LightCycler® 480 system (Roche Diagnostics GmbH, Germany) using the Roche LightCycler® 480 SYBR Green I Master Mix, 2µl of 1:5 diluted cDNA and 10µM of each primer. The primer design was done with the programme PerlPrimer. Three housekeeping genes (GAPDH, RPS13, SUZ12) were used to normalise the absolute transcript numbers. Then the array results of the chosen genes were correlated with the corresponding qRT-PCR values by calculating the respective Pearson coefficients (ORIGIN® 8.0 by ORIGINLab), the significance level set at $p < 0.05$.

3 Results and discussion

Figure 1 shows an overview over the numbers of significantly regulated probe sets between the different groups at the marked timepoints, also marking the overlap between them. The regulation in both groups between the timepoints outweighs the regulation at both timepoints

between the groups. It's notable that the regulated non-overlapping transcripts of the fertile group are about double the number of those of the infertile group.

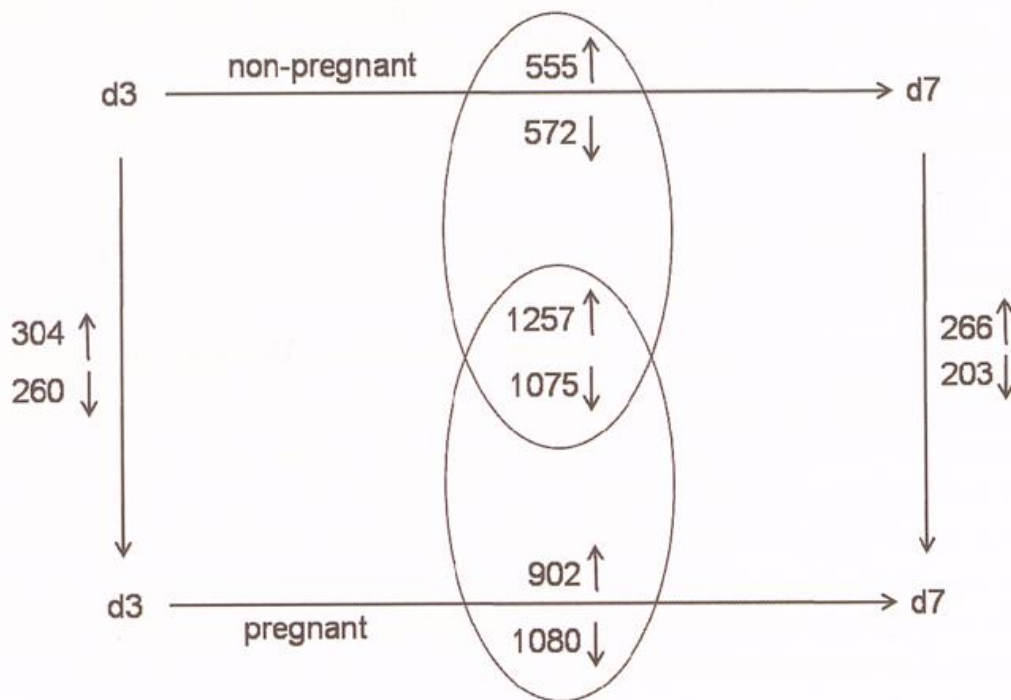


Figure1: number of up- and down-regulated transcripts in the endometrium of differently fertile heifers at different timepoints (days 3 and 7 of the estrous cycle, respectively)

The IPA between the non-pregnant vs. the pregnant group on day 3 revealed a regulation of amongst others several cell cycle pathways and estrogen receptor signalling. Estrogen is a key element in development and fertility and acts on a variety of other genes, so the up-regulation of this pathway in the pregnant group indicates indeed a direct connection between the transcriptional differences and the pregnancy success. Day 7 was characterised by the regulation of pathways mainly related to cellular proliferation and development, one of the most prominent was IGF-1 signalling.

From the 10 genes selected to validate the microarray results, 8 showed a stringent positive correlation (the exceptions being DNMT3A and ASMT). Two of these genes had been picked due to prior literature research regarding their function instead of according to their significant regulation on the arrays like the others. Both of these – CBR1 and PTGES – are prostaglandin synthases. Prostaglandins (PGs) are tissue hormones derived from arachidonic acid and act amongst other things as primary regulators of female reproductive function; they are regulated by a complicated balance of receptors, catabolic enzymes and synthases.

In ruminants, the establishment of pregnancy depends heavily on the PG-E₂/PG-F_{2α} ratio, with PG-E₂ operating luteoprotective and PG-F_{2α} acting as its luteolytic antagonist. CBR1 has been described as the PG-F_{2α} [08] and PTGES as the PG-E₂ [09] synthase in bovine endometrium. Neither of the two was found regulated in our experiment, though other prostaglandin calibrators were – e.g. HPGD, an enzyme involved in the inactivation of PG-E₂ and PG-F_{2α}, was found to be significantly lower expressed in the group of fertile heifers on day 7, confirming the importance of a finely tuned balance in the prostaglandin system for the establishment of pregnancy.

4 Acknowledgements

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Regulation of glucose and lipid metabolism during transition in high yielding dairy cows with differences in fat mobilisation

Claudia Weber¹, Cornelia Hametner¹, Frank Becker¹, Bernd Losand², Armin Tuchscherer¹, Rupert M. Bruckmaier³, Wilhelm Kanitz¹, and Harald M. Hammon¹

¹Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, ²State Institute for Agriculture and Fishery, Dummerstorf, Germany, ³Veterinary Physiology, University of Bern, Switzerland

1 Introduction

Dairy cows mobilise body fat during early lactation to provide energy for milk production due to insufficient feed intake. However, there is huge individual variation in postnatal fat mobilisation among cows indicated by a broad range of postnatal liver fat concentrations (LFC) (Hammon et al., 2009). The ability to cope with metabolic stress during the transition period varies among individual cows under similar environmental conditions, therefore a genetic background is presumed to explain the differences in adaptation to lactation (Kessel et al., 2008). A lot of studies are available that have investigated metabolic adaptations and hepatic gene expression of enzymes involved in energy metabolism during the transition period, but less studies consider the interindividual variations within breed and lactation number. This study was conducted to investigate interindividual variations in feed intake, body condition, metabolic parameters as well as gene expression of hepatic enzymes involved in glucose metabolism during the transition from gestation to lactation in high yielding dairy cows differing in their fat mobilisation after calving. Classification of fat mobilisation was indicated by changes in liver fat concentrations after calving.

2 Materials and methods

German Holstein cows (in average 11,500 kg milk/305 d in 2nd lactation) were grouped according to different mean LFC on d 1, 14 and 28 after calving: **L** (low; <200 mg total fat/g DM; n=10), **M** (middle; 200-300 mg total fat/g DM; n=10), and **H** (high; >300 mg total fat/g DM; n=7). Cows were studied from dry off (8 weeks before expected calving) up to 63 days in milk (**DIM**) in their 3rd lactation and were fed TMR *ad libitum* accordingly to the recommendations of German Society of Nutritional Physiology (2001). Dry matter intake (**DMI**) and milk yield (**MY**) were recorded daily, body weight (**BW**), body condition score (**BCS**), back fat thickness (**BFT**) and milk composition were measured once weekly. Energy balance (**EB**) was calculated as: $EB = NE_L\text{-intake} - (ECM \times 3.14 + 0.293 \times \text{kg BW}^{0.75})$ (Reist et al., 2002) and was expressed in MJ/(d × cow). Energy-corrected milk (**ECM**) was calculated as follows: $ECM \text{ (kg)} = (0.038 \times \text{g crude fat} + 0.024 \times \text{g crude protein} + 0.017 \times \text{g lactose}) \times \text{kg milk}/3.17$ (Reist et al., 2002). Plasma concentrations of non-esterified fatty acids (**NEFA**), beta-OH-butyrate acid (**BHBA**), glucose and insulin were measured in blood taken at 56, 28, 15, 5 d before expected calving, 1 d after calving, and once weekly up to 63 DIM. Liver biopsies were taken at 56 and 15 d before calving and 1, 14, 28, 49 DIM to measure total fat

concentrations, glycogen concentrations and hepatic mRNA concentrations of enzymes involved in glucose metabolism (pyruvate carboxylase (**PC**), cytosolic phosphoenolpyruvate carboxykinase (**PEPCKc**), glucose-6-phosphatase, propionyl-CoA-carboxylase (**PCCA**)). Data were analyzed by the Mixed Model of SAS with LFC and time as fixed effects. For all data calculations were done for several time intervals: prepartum (8 wks before parturition until calving), transition (2 wks before until 4 wks after calving), postpartum (0 to wk 9), and the entire observation period from wk 8 before until wk 9 after calving. Data are presented as LSMeans $\pm$ pooled SE for the whole experimental period.

3 Results

Mean hepatic fat concentrations for H, M and L were different ($P < 0.01$) among groups: 351 ± 14 , 250 ± 10 and 159 ± 9 mg/g liver DM, for H, M, and L, respectively. DMI increased ($P < 0.01$) after calving in all groups, but was highest ($P < 0.05$) in L during transition and early lactation. MY and ECM did not differ among groups, thus energy balance during transition and early lactation was least negative ($P < 0.01$) in L. BFT decreased after calving much less ($P < 0.05$) in L than in H and M (Figure 1). Plasma concentrations of NEFA and BHB increased more around calving ($P < 0.05$) in H than M and L, but plasma glucose was lowest in H ($P = 0.05$) (Figure 2). Cows in L showed higher plasma insulin concentrations ($P < 0.05$) than H and M cows during the post calving period. Liver glycogen concentrations decreased more in H ($P < 0.05$) than L and M after calving (data not shown). The mRNA concentrations of selected enzymes, except PCCA, changed with time during the experimental period (Figure 3). PC mRNA concentrations increased immediately after calving highest ($P < 0.05$) in H and M. Levels for PEPCKc mRNA tended to be highest in L ($P < 0.1$) immediately after calving.

Figure 1: Zootechnical parameters in low (L) ($\blacktriangle$), middle (M) ($\circ$), high (H) ($\square$)

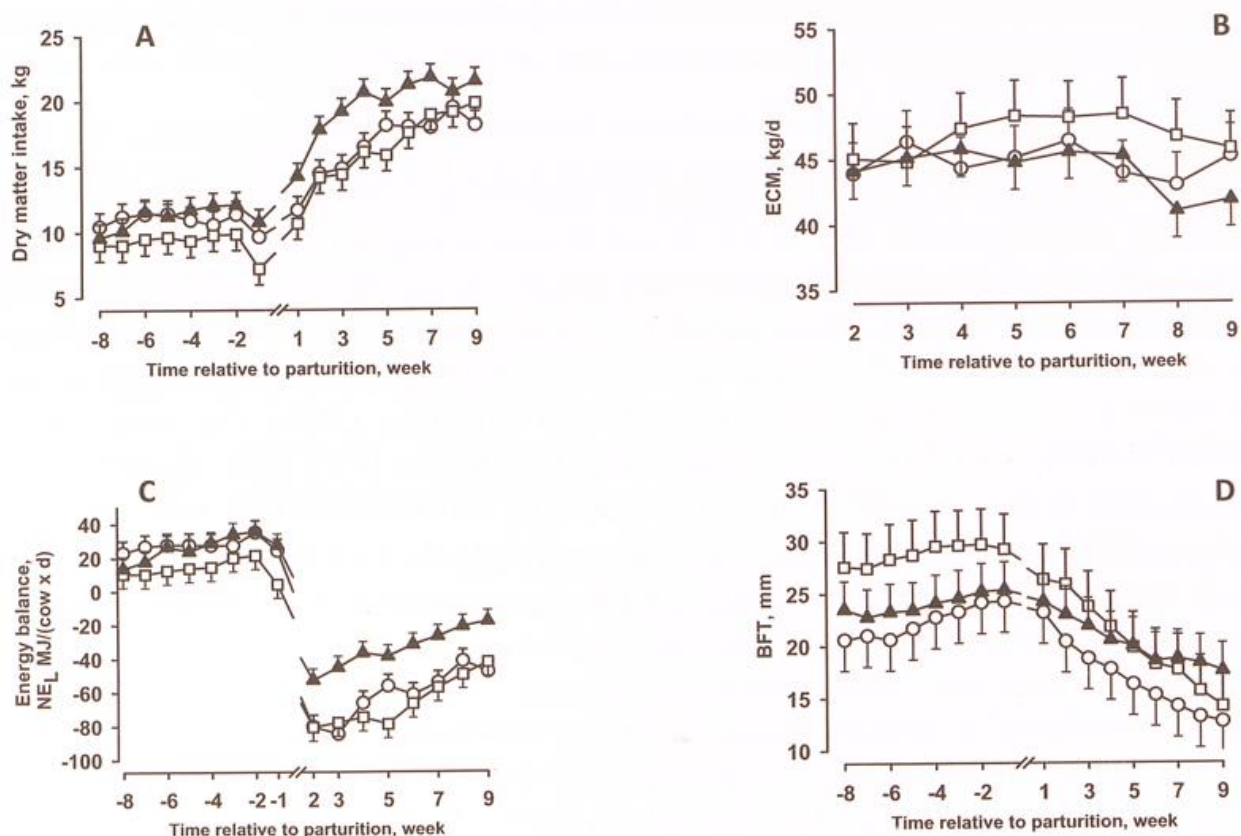


Figure 2: Metabolites and Insulin in low (L) ($\blacktriangle$), middle (M) ($\circ$), high (H) ($\square$)

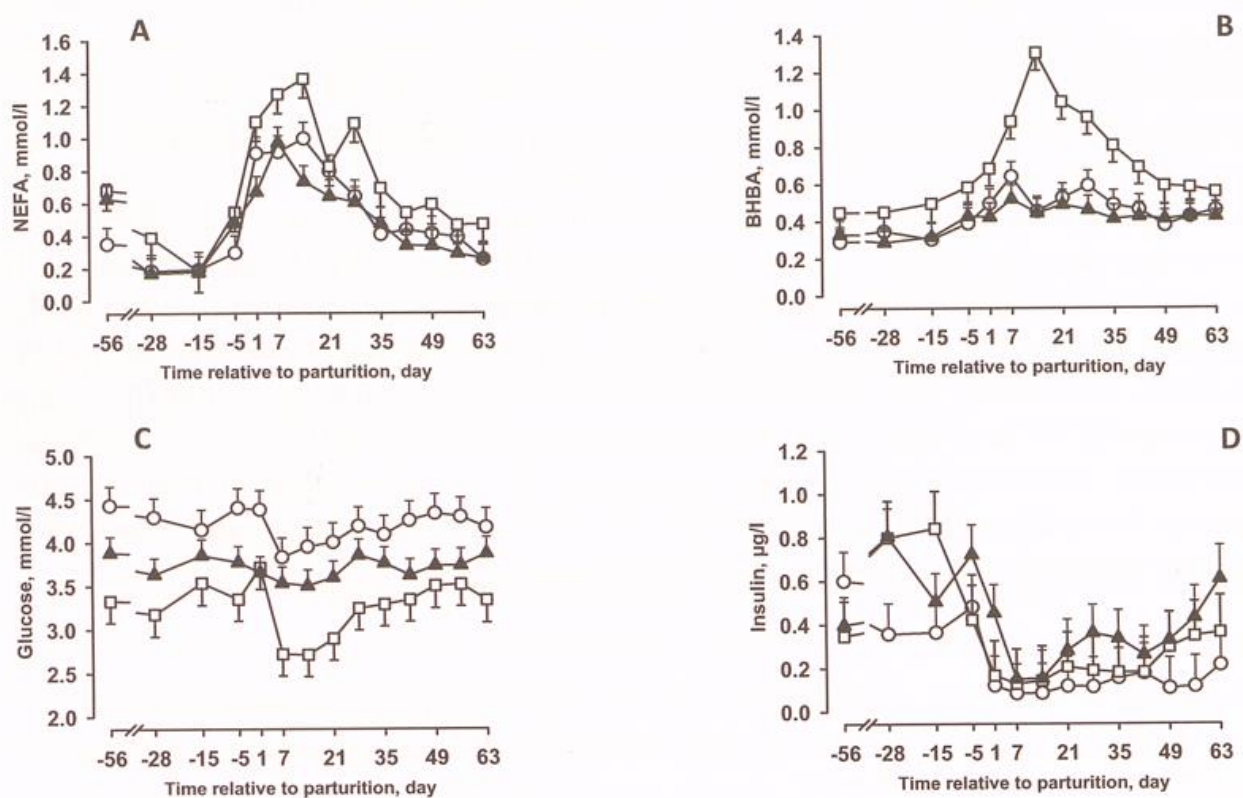
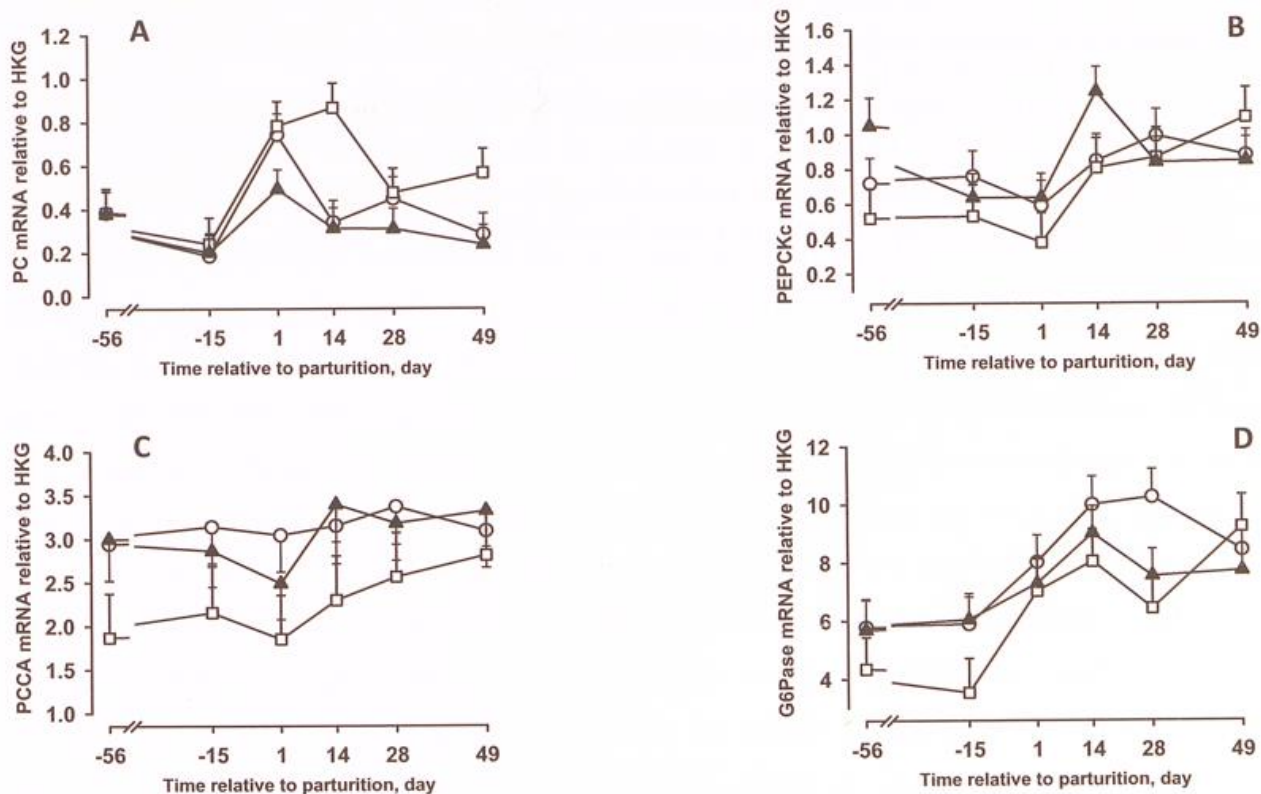


Figure 3: mRNA concentrations of enzymes relative to housekeeping gene (GAPDH) in low (L) ($\blacktriangle$), middle (M) ($\circ$), high (H) ($\square$)



4 Discussion

Greater fat mobilisation after calving was associated with reduced DMI and lower energy balance, but did not affect milk production, as also seen by Hammon et al. (2009). Elevated mobilisation of subcutaneous fat layer during early lactation did not depend on pre-calving body condition and BFT, because BFT decreased in H and M cows in the same way, although body condition and BFT before calving was greater in H than M. On the other hand, BFT immediately before calving was similar in L and M cows, but after calving M cows mobilised more subcutaneous fat than L cows. Higher plasma NEFA and BHBA concentrations in H corresponded to highest LFC in this group. On the other hand, postnatal glucose and insulin status as well as hepatic glycogen concentrations were reduced in H. Gene expression of PC increases immediately after calving, as shown before by Greenfield et al. (2000). Greater expression of PC in H and M was probably the consequence of a shift in using more lactate and amino acids and less propionate as precursor for gluconeogenesis immediately after calving due to lower DMI in H and M cows. In conclusion, high-yielding dairy cows show variable metabolic responses to lactation stress by adapting fat and glucose metabolism.

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Effect of maternal protein consumption during gestation on the ontogenetic development of the proteome profile in liver and muscle of IUGR porcine offspring

Martin Peters¹, Björn Kuhla¹, Iris S. Lang¹, Paul Eberhard Rudolph² and Cornelia C. Metges¹

Research Unit ¹Nutritional Physiology and ²Genetics and Biometry, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

1 Introduction

Common and central feature of intrauterine growth restriction (IUGR), a symptomatic complex correlating with increased perinatal morbidity and mortality (Seeds, 1984) as well as comprised postnatal development (Litten et al., 2003), is the impairment of fetal oxygen and nutrient supplementation. The reduced growth rate as a consequence of intrauterine malnutrition represents a sensible adaptation process to enable fetal survival (Warshaw, 1985) although the exact cause of IUGR seems to be not as important as timing and extend of the insufficient nutrient supply (Wollmann, 1998). Excessive or deficient nutrient supply studies with rodents already proved direct programming effects (Lillicrop et al., 2007) leading to an increased interest in similar research studies on farm animals. The objective for this study was to determine the ontogenetic development of the proteome profile in liver and muscle of IUGR porcine offspring to explore mechanisms of adaptation and growth related to inadequate maternal dietary protein supply during gestation.

2 Materials and methods

The subset of animals used in this study was generated in experiment 1 published by Rehfeldt et al. (2011) and comprised 242 pig offspring of both genders born to 50 gilts fed isocaloric maternal diets differing in protein concentration (low protein, LP, 6.5% CP; adequate protein, AP, 12.1% CP; high protein, HP, 30% CP). Offspring liver and M. longissimus dorsi were collected at 1, 28 and 188 days post natum (dpn). Piglets were classified in groups of low (0.98 ± 0.02 kg, $n = 45$) and high ($1.57 \text{ kg} \pm 0.02$ kg, $n = 42$) birth weights and growing pigs were divided in groups of lower (28. dpn: 1.15 ± 0.10 kg, $n = 42$; 188. dpn: 1.15 ± 0.10 kg, $n = 37$) and higher birth weight (28. dpn: 1.45 ± 0.12 kg, $n = 36$; 188. dpn: 1.45 ± 0.12 kg, $n = 40$). Sample preparation, two dimensional (2D) proteome and image analysis, peptide mass fingerprinting and Western Blot for liver tissue was similar to methods used for cow liver (Kuhla et al., 2009). Sample preparation for muscle proteome analysis included the use of a protease inhibitor cocktail and was modified in respect to sample amounts, centrifugation time, and protein load per gel for optimized 2D electrophoresis separation

results. Considering gender (male/female), birth weight (light/heavy) and diets fed to the gilts (AP/HP/LP), neonatal piglets used for tissue collection were allocated in twelve sample categories. To ensure similar distribution of observations in each group, sample extracts of up to three piglets from identical gender, gilt diet, and birth weight classes were pooled. If tissue samples needed to be pooled, the total protein load per gel of $x \mu\text{g}$ was achieved by pooling $x/2 \mu\text{g}$ of two or $x/3 \mu\text{g}$ of three liver extracts. Relative spot volumes were compared using a three-way ANOVA and SAS procedure MIXED (SAS software version 9.1.3 (SAS, 2004) with the main factors gilt diet, gender, and birth weight, and the interactions diet $\times$ birth weight and diet $\times$ birth weight $\times$ gender. For birth weight analysis, three-way ANOVA and SAS procedure GLM (SAS software version 9.1.3 (SAS, 2004) with identical main factors and interactions was used for comparison. Post hoc comparisons between relative spot volumes were made using the Tukey-Kramer test. Spots showing significant differences at $P \leq 0.05$ and $> 20\%$ up or down regulation in protein expression of HP and LP compared to AP offspring or between birth weight class comparisons were chosen for protein identification by MALDI-TOF mass spectrometry analysis. Differences in protein expression among groups were indicated as fold change with ratios of the least square means smaller than 0.8 and greater than 1.2 (versus AP) taken into consideration.

3 Results

LP and HP piglets at 1 and 28 dpn showed a reduction in birth weight ($P < 0.01$) compared to AP piglets, whereas the birth weight of pigs slaughtered at 188 dpn was only reduced in the LP group compared to the AP and HP group (Table 1).

Table 1 Birth weight (kg) of porcine offspring chosen for proteome analysis (1.dpn, $n=27/\text{LP}$, $n=28/\text{AP}$, $n=32/\text{HP}$; 28. dpn, $n=25/\text{LP}$, $n=24/\text{AP}$, $n=29/\text{HP}$; 188.dpn, $n=24/\text{LP}$, $n=26/\text{AP}$, $n=27/\text{HP}$)

	Diet ¹			P value ²					
	LP	AP	HP	D	BWC	G	DxBWC	DxG	DxBWCxG
1. dpn	$1.22^a \pm 0.03$	$1.35^b \pm 0.03$	$1.26^a \pm 0.03$	0.007	<0.001	0.684	0.466	0.596	0.030
28. dpn	$1.20^a \pm 0.02$	$1.46^b \pm 0.02$	$1.24^a \pm 0.02$	<0.001	<0.001	0.482	0.186	0.169	0.232
188. dpn	$1.20^a \pm 0.02$	$1.40^b \pm 0.02$	$1.34^b \pm 0.02$	<0.001	<0.001	0.351	0.270	0.275	0.788

¹Values are LSMs $\pm$ SD per diet over 2 birth weight classes and 2 genders.

²ANOVA PROC GLM, D = Diet, BWC = Birth Weight Class (L = Light or H = Heavy), G = Gender (M = Male or F = Female)

^{a,b} Within a row, LSMs not sharing a common superscript differ significantly ($P < 0.05$) according to Tukey post hoc test.

During proteome analysis at 1, 28 and 188 dpn, in liver 125, 192 and 96 spots were identified from a total of 988, 791 and 906 detected spots, coding for 77, 86 and 51 differentially expressed proteins, respectively. Out of 1135 spots detected in muscle samples taken at 1, 28 and 188 dpn 264, 156 and 54 spots were identified yielding a total of 109, 79 and 33 differentially expressed proteins, respectively. In total, 785 liver spots and 384 muscle spots were picked for peptide mass fingerprinting. Liver or muscle proteins encoded for by 2 spots or more and demonstrating a differential expression level of at least 20% are summarized according to their main effect (maternal protein consumption during gestation or birth weight class) in Table 2. Low maternal protein consumption during gestation

increased hepatic indicators of oxidative stress (AHSG, HSP90B1, HSPA5) and protein degradation (VCP) probably due to reduced transport levels of nutrients and oxygen (ALB, HBA1) in neonatal piglets. Elevated hepatic stress in HP piglets is still detectable around weaning (28 dpn) accompanied by urea cycle activation (CPS1). At 188 dpn xenobiotic (ADH1A, GSTM2) and other catabolic pathways (CES2, HPD) are induced while protein methylation (BHMT) was decreased. Porcine offspring of gilts fed HP diet during gestation exhibited increased hepatic glycolytic (GRHPR) and other energy dependent enzymes (GLUD1) at birth but lower expression levels of these enzymes (PGK1, GLUD1, ATP5B) at weaning. In contrast to LP pigs at 28 dpn hepatic stress levels are reduced (HSP90B1, HSPA13) in HP pigs at this age probably due to the higher rate of correct protein folding (CALR) and intracellular calcium signalling (RGN). At the age of 188 dpn, hepatic protein folding is still increased (P4HB, PDIA3) but protein methylation is decreased (BHMT) resulting in higher stress levels (HSPA5) and platelet activation (FGA).

Table 2 Differential expression of proteins in liver and muscle due to maternal protein consumption during gestation or birth weight class

		Diet ¹		
		LP vs AP	HP vs AP	L vs H
Liver	1. dpn	ACTB (1.37), AHSG (1.29), AKR1A1 (1.50), ALB (0.71), ATP5B (1.46), HBA1 (0.65), HSP90B1 (1.44), HSPA5 (1.28), VCP (1.36)	FTH1 (1.66), ISYNA1 (0.54), GLUD1 (1.42), GRHPR (1.27)	FTH1 (1.43), ISYNA1 (2.04), ENO3 (1.49), GAPDH (0.75), HP (1.32), HSP90AA1 (1.56)
	28. dpn	CPS1 (1.22), GLUD1 (0.63), HSP90B1 (1.70), HSPA5 (1.20), PRDX2 (0.74)	CAT (0.75), GLUD1 (0.67), RGN (1.91), ATP5B (0.68), CALR (1.21), ETFA (0.63), HSP90B1 (0.76), HSPA13 (0.54), KRT8 (0.72), PGK1 (0.70)	ALB (0.80), TF (1.70), FGA (0.71), HSPD1 (1.22), QDPR (1.22)
	188. dpn	ALB (1.50), CES2 (1.40), ADH1A (1.49), BHMT (0.80), GSTM2 (1.34), HPD (1.27)	HSPA5 (1.28), ALB (1.56), FGA (1.39), BHMT (0.79), P4HB (1.28), PDIA3 (1.33)	CES2 (1.64)
Muscle	1. dpn	ACTA1 (1.23), ALB (0.64), MYH4 (3.36), TNNT3 (0.80)	HBB (1.45), TNNT3 (1.34), ACTA1 (1.48), HSPA5 (1.33), P4HB (0.74), VCP (1.44)	ACTN2 (1.95), DES (1.88), ENO3 (0.73), ALDOC (0.66), CA3 (0.65), IDH3A (1.49), MYOZ1 (1.65), TP11 (0.77), VCL (2.49), VIM (1.72)
	28. dpn	CKM (1.26), MYL3 (0.69), ENO3 (1.22), MYBPC2 (0.43), ALDOC (0.46), CA3 (0.71), HBB (0.51), LDB3 (0.65), MYLPF (0.57), VCL (0.36), VCP (0.48)	MYL3 (0.54), HBB (0.33), DES (1.38), HSP90B1 (0.77), LDB3 (0.53), MYBPC2 (0.23), MYLPF (0.42), VCL (0.26), VCP (0.47)	HSP90B1 (1.45), TF (1.38), TNNT3 (1.53), TP11 (1.28), ACO2 (1.40), CKM (1.57), DES (1.78), FABP1 (1.34)
	188. dpn	ENO3 (0.67), MYL3 (1.32)	ALB (0.67)	MYH4 (0.58)

¹Values are given as ratios (r) of the LSMs (r = LP/AP, r < 1 = LP < AP, r > 1 = LP > AP; r = HP/AP, r < 1 = HP < AP, r > 1 = HP > AP; r = L/H, r < 1 = L < H, r > 1 = L > H; r = M/F, r < 1 = M < F, r > 1 = M > F)

ACO2=aconitase 2, ACTA1=actin alpha 1, ACTB=actin beta, ACTN1=actinin alpha 1, ADH1A=alcohol dehydrogenase 1A, AHSG=alpha-2-HS-glycoprotein, AKR1A1=aldo-keto reductase family 1 A1, ALB=albumin, ALDOC=aldolase C, ATP5B=ATP synthase, BHMT= betaine-homocysteine S-methyltransferase, CA3=carbonic anhydrase III, CALR=calreticulin, CAT=catalase, CES2=carboxylesterase 2, CKM=creatine kinase, CPS1=carbamoyl-phosphate synthase 1, DES=desmin, ENO3=enolase 3, ETFA=electron-transfer-flavoprotein alpha, FABP1=fatty acid binding protein 1, FGA=fibrinogen alpha, FTH1=ferritin heavy polypeptide 1, GAPDH=glyceraldehyde-3-phosphate dehydrogenase, GLUD1=glutamate dehydrogenase 1, GRHPR=glyoxylate reductase/hydroxypyruvate reductase, GSTM2=glutathione S-transferase mu 2, HBA1=hemoglobin alpha 1, HBB=hemoglobin beta, HP=haptoglobin, HPD=4-hydroxyphenylpyruvate dioxygenase, HSP90AA1=heat shock protein 90kDa alpha A1, HSP90B1=heat shock protein 90kDa beta, HSPA5(13)=heat shock 70kDa protein 5(13), HSPD1=heat shock 60kDa protein 1, IDH3A=isocitrate dehydrogenase 3 alpha, ISYNA=inositol-3-phosphate synthase 1, KRT8=keratin 8, LDB3=LIM domain binding 3, MYBPC2=myosin binding protein C, MYH4=myosin heavy chain 4, MYL3=myosin light chain 3, MYLPF=myosin light chain phosphorylatable, MYOZ1=myozenin 1, P4HB=prolyl 4-hydroxylase beta, PDIA3=protein disulfide isomerase A3, PGK1=phosphoglycerate kinase 1, PRDX2=peroxiredoxin 2, QDPR=quinoid dihydropteridine reductase, RGN=regucalcin, TF=transferrin, TNNT3=troponin T3, TP11=triosephosphate isomerase 1, VCL=vinculin, VCP=valosin containing protein, VIM=vimentin

The predominant increase of structural filaments (ACTA1, MYH4) in skeletal muscle of LP neonatal piglets was reversed (MYL3, MYBPC2, LDB3, MYLPF, VCL) at 28 dpn and again higher (MYL3) at 188 dpn. Similar to the protein expression pattern of LP skeletal muscle the marked increase of structural filaments (TNNT3, ACTA1) in neonatal piglets is reversed at weaning (MYL3, LDB3, MYBPC2, MYLPF, VCL), a development which is also true for increased levels of oxygen transport (HBB), markers of oxidative stress (HSPA5, HSP90B1) and proteolytic activities (VCP). The impaired transport capacity continues up to 188 dpn (ALB). Low birth weight independent of maternal protein consumption increased hepatic iron dependent proteins (FTH1, HP), markers of oxidative stress (HSP90AA1) and the production of all inositol-containing compounds (ISYNA1), including phospholipids in neonatal piglets. At 28 dpn hepatic protein folding and amino acid breakdown were increased probably reflecting earlier malnutrition (ALB). Skeletal muscle of low birth weight offspring exhibits increased structural filament formation (ACTN2, DES, MYOZ1, VCL, VIM, TNNT3) at 1 dpn and weaning but lower levels of these proteins at the age of 188 dpn (MYH4). Glycolytic enzyme levels (ENO3, ALDOC, TPI1) in skeletal muscle were reduced in neonatal piglets but increased at weaning (TPI1) as well as other enzymes related to energy supply (ACO2, CKM).

4 Discussion

Piglet birth weight was similar to the birth weight of porcine offspring observed in the complete set of experimental animals (Rehfeldt et al., 2011), validating our animal subset as a model for IUGR. Concluding from proteome results, maternal LP and HP diets changed the offspring proteome profile of major metabolic pathways, although changes seem not to be persistent but vary depending on the age of the pigs. Only structural filament remodelling in skeletal muscle tissue was homogenously influenced in LP and HP offspring at 1 and 28 dpn, indicating similar regulation patterns independent of maternal protein consumption during gestation. However, the mostly different diet-dependent profiles indicate different intrauterine regulatory mechanisms leading to a similar offspring IUGR phenotype. This demonstrates the functional plasticity of growing pigs.

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Influence of lactation performance on muscle composition and meat quality in SEGFAM-F2 cows

Mohamed Hamada^{1,2}, Elke Albrecht¹, Abdel-Rahman El Bagory², Abo-Bakr Edris³, Gerd Nuernberg⁴, Harald M. Hammon⁵ and Steffen Maak¹

¹Leibniz Institute for Farm Animal Biology (FBN), Research Unit Muscle Biology and Growth, Dummerstorf, Germany

²Minufia University, Department of Food Hygiene & Control, Faculty of Veterinary Medicine, Minufia, Egypt

³Banha University, Department of Food Hygiene & Control Faculty of Veterinary Medicine, Banha, Egypt

⁴Leibniz Institute for Farm Animal Biology, Research Unit Genetics and Biometry, Dummerstorf, Germany

⁵Leibniz Institute for Farm Animal Biology, Research Unit Nutritional Physiology, Dummerstorf, Germany

1 Introduction

Beef and dairy cattle represent divergent metabolic types that disseminate nutrients preferably into either meat or milk and differ in nutrient accretion [1]. To investigate effects of variability in lactation performance on carcass and meat quality traits in cows having a defined, combined beef and dairy genetic background, 50 cows with varying lactation performance were selected from F₂ offspring of the SEGFAM experiment [2] at the FBN, Dummerstorf. The capacity for milk production is the main metabolic driving force; so differences in milk production are accompanied by differences in body composition [3]. Consequently, carcass and meat traits differ in relation to lactation performance [4; 5]. Lactation is a physiological state associated with significant metabolic adaptations and changes in energy balance [6]. To meet the increased energy demands for milk synthesis, cows can mobilize body reserves [7]. A possible indicator of fat mobilization is the fat cell diameter [8]. In high lactating cows, also muscle degradation can be involved in restoring energy balance in conjunction with lipolysis and an increase in appetite [9]. The objective of the presented study was to test the hypothesis that fat cell and muscle fibre size, muscle fibre type composition and consequently carcass composition and meat quality traits are influenced by lactation performance in cows with a combined beef and dairy genetic background.

2 Material and methods

Animals and sampling: A sample of 50 cows was selected from the SEGFAM F2 resource population [2] and assigned to 3 groups according to their milk yield during the first lactation. The groups were named high lactating (HL, n = 16), low lactating long (LLL; duration of first lactation >100 days; n = 19), and low lactation short (LLS; duration of first lactation <100

days; n = 15). Cows were slaughtered 30 days after calving during the second lactation. Samples of *longissimus dorsi* (LD) muscle and subcutaneous fat (SCF) were taken for histology immediately after slaughter from the left side of the dressed carcass, snap frozen in liquid nitrogen, and stored at -70°C until use. Samples for determination of meat quality were taken after chilling at 4°C for 24 h. A 2 to 3 cm thick slice of LD was prepared for marbling evaluation.

Carcass & meat quality traits: Carcass traits including body weight (BW), hot carcass weight (HCW), cold carcass weight (CCW), weights of different fat depots, and cold carcass fat and protein were determined as described by Pfuhl et al. [10]. Meat quality data were determined for the LD using standard procedures; including LD weight and dimensions, pH value, colour, water binding capacity, Warner-Bratzler shear force at 24 h and 14 d *pm*, and intramuscular fat content.

Histology and image analysis: For assessment of marbling traits, fixed LD slices were stained with Oil Red-O and prepared for computer image analysis as described by Albrecht et al. [11]. For fat cell size determination (either in LD or in SCF), frozen samples were cut and stained with haematoxylin and eosin (H/E). Marbling and fat cell size were measured using an image analysis system as described by Albrecht et al. [12]. For muscle fibre typing and determination of fibre size and number of nuclei per muscle fibre, serial sections were stained with H/E and reacted for ATPase stability to allow a clear discrimination between fibre types. Muscle fibre size and types were measured and classified with an image analysis system according to Albrecht et al. [12].

Statistical analysis: Statistical analysis was performed using the SAS statistical software (version 9.2, SAS Inst. Inc. 2009, Cary, USA). Data were analysed by ANOVA using a mixed model containing a covariate age at slaughter or parturition and fixed factors group, family, and group x family interaction. Pearson correlation coefficients were calculated to determine the relationships between milk yield and carcass traits, meat quality, or muscle structure using the CORR procedure of SAS.

3 Results and discussion

Milk yield: Milk yield was generally low in the first lactation period, but significantly different between the groups. Possibly, animals might have been not physically mature and they required nutrients for their own continued growth [13; 14]. Low milk yield in both lactation periods is consistent with the low production of Charolais cows, but was too low

when compared with German Holstein cows [15]. The amount of milk per day in the first lactation was influenced by age at parturition ($P < 0.01$) in consistence with [16]. The duration of the first lactation period differed ($P < 0.01$) among the three groups being $HL > LLL > LLS$ (283.9, 111.9, and 47.6, respectively). The amount of milk per day (Fig. 1), however, was similar in both low lactating groups and higher in HL ($P < 0.01$) in both lactation periods.

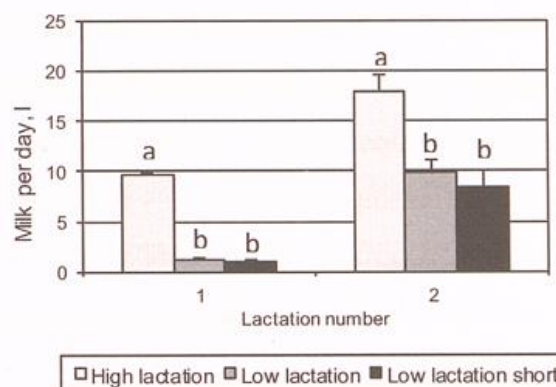


Fig. 1: Milk yield of F₂ cows. Letters indicate significant differences between groups ($P < 0.01$).

Carcass characteristics: Analysis of variance revealed no difference between LLL and LLS group of cows for none of the investigated traits. Therefore these groups were considered together as LL group to investigate the influence of amount of milk per day on carcass traits. Milk yield differences were accompanied by differences in body weight and carcass composition. Cows of the LL group were heavier (774 vs. 715 kg; $P < 0.05$) and achieved higher carcass yields (55.3 vs. 52.5%; $P < 0.01$) than HL cows. The LL cows tended to store more internal fat (41.3 vs. 49.8 kg; $P = 0.06$) and the carcasses of LL cows contained more protein (55.9 vs. 49.5 kg; $P < 0.01$). These results suggest differences in utilization of nutrients for either milk secretion or tissue accretion.

Meat quality: Differences in milk yield had only minor effects on LD meat quality. The muscle weight was greater in LL cows (8.3 vs. 7.3 kg; $P < 0.02$), accompanied by a slightly larger cross sectional area (87.7 vs. 77.9 cm²; $P = 0.05$). Fat content, water binding capacity, and shear force were not different between groups ($P > 0.09$). The pH value 24 h after slaughter, however, was lower in LD of HL cows (5.59 vs. 5.52; $P = 0.01$). Marbling traits were not different between groups ($P > 0.4$), except the size of marbling flecks, which was larger (0.88 vs. 0.65 mm²; $P = 0.01$) in LD of LL cows.

Fat cell size and muscle structure: Muscle fibre size and composition of MLD as well as fat cell profile were not influenced by lactation performance. The fat cell size was similar in both groups ($P > 0.3$) with an average diameter of 60 µm and 86 µm for intramuscular and subcutaneous fat cells respectively. Between HL and LL cows, there was neither significant difference in muscle fibre size nor in muscle fibre type composition ($P > 0.06$). On the other hand, the number of nuclei per muscle fibre was significantly increased ($P < 0.04$) in total (1.47 vs. 1.26), fast (1.66 vs. 1.45), and intermediate (1.56 vs. 1.28) muscle fibres of HL cows. This might be attributed to adaptation of skeletal muscle fibres to changes in function, which can

alter the total number of myonuclei [17]. No indication for fat or protein mobilization from muscle or SCF was found, probably because cows were not in a negative energy balance as assumed for other F2 heifers of the same experiment [18].

Relationships between traits were generally low or not significant, but showed consistently that increasing milk yield was negatively correlated with tissue accretion. Consequently, cows of the LL group gained more weight and stored more fat than HL cows and reached higher carcass yields. Muscle structure and meat quality were not influenced by lactation performance.

The results suggest that cows of both groups utilized nutritional energy for lactation and tissue accretion to a different extent.

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Complete results of the study will be published elsewhere.

IDO activation by LPS stimulation: physiological consequences and their blocking by 1-methyl-tryptophan

Elisa Wirthgen, Ellen Kanitz, Margret Tuchscherer and Winfried Otten

Leibniz Institute for Farm Animal Biology (FBN), Research Unit Behavioural Physiology

1 Introduction

Indoleamine 2, 3-dioxygenase (IDO) is an initial and rate limiting enzyme for the kynurenine (Kyn) pathway of tryptophan (Trp) degradation. In this pathway, Trp is degraded to biological active metabolites like Kyn, quinolinic acid (Quin) and kynurenic acid (Kyna). IDO is expressed ubiquitously in a constitutive or inducible manner (1). In many cell types it is induced by specific inflammatory stimuli like interferon- γ (IFN- γ) or lipopolysaccharides (LPS) (2). Recent studies have shown that IDO activation is one mechanism of the innate immunity and has different physiological functions with a highly cell-type specific pattern of inducibility (1). The depletion of Trp by IDO induction is an antiproliferative mechanism leading to the suppression of various intracellular pathogens and T-cell proliferation (3, 4). Currently, many findings about the biological function of IDO are coming from *in vitro* studies in murine or human cells. Concerning the high relevance of IDO in immunological and neurological disorders (3), we still need a better understanding of IDO as a part of physiological or even pathogenic processes *in vivo*. The pig is a suitable model for human diseases because of the high similarity of human and porcine physiology. In addition, a better knowledge about the role of the Trp metabolism in immune responses of pigs could help to improve an appropriate animal husbandry. The present study comprises two main work steps. The first part aims at characterizing *in vivo* effects of LPS on IDO expression, Trp metabolism and physiological parameters like cytokines and hormones. LPS, as a strong immune stimulant, is used as a model for IDO activation. In the second part IDO activity is blocked with the specific inhibitor 1-methyl-tryptophan (1-MT), which enables us to determine the specific role of IDO in the network of the immune response and potential therapeutic effects of 1-MT.

2 Materials and methods

All experiments were performed with seven-week-old male castrated German Landrace pigs bred and raised in the experimental pig unit of the FBN.

In vivo LPS administration. IDO activity was induced by a single intraperitoneal LPS administration (100 µg/kg body weight). The LPS dose was determined in previous studies and induced sickness behaviour like shivering and a significant increase of plasma tumor necrosis factor- α (TNF- α) and kynurenine concentrations. Blood samples were taken by a puncture of *vena cava cranialis* before LPS and 1, 3, 6 and 24 h after LPS administration. Cytokines TNF- α , interleukin-10 (IL-10) and IFN- γ were analyzed by using commercial available pig ELISA kits. TNF- α and IL-10 were determined in blood plasma. IFN- γ was measured in supernatant of whole blood culture after stimulating the blood cells with staphylococcal enterotoxin B (SEB) for 24 hours. Trp and its metabolites were measured in blood plasma using tandem mass spectrometry (MS/MS) at the University of Greifswald. Cortisol was analyzed by a commercial available human ELISA kit, validated for porcine plasma. Protein expression of IDO was detected by western blot analysis using a polyclonal antibody against human IDO. Anti-Actin antibody was used as a loading control.

A model for blocking of IDO with 1-MT. 1-MT was solved in a triglyceride (TG) solution and injected subcutaneously (s.c.) (1 g/day). Animals with injections of the TG solution alone were used as control group. The 1-MT absorption after a single injection was determined in blood samples of catheterized pigs over a period of one week. Based on this kinetics, a model for repeated injections was created. The 1-MT solution was injected daily over a period of four days (1g 1-MT/day). Blood samples were taken before every 1-MT application for analyzing 1-MT per MS/MS.

Statistics. The data of all experiments were evaluated by analyses of variance (ANOVA) using the mixed procedure including repeated measurements for each animal. The model comprised the main effects treatment, time, replication and their two way interactions. For pairwise comparisons the Tukey–Kramer procedure was used. Differences were considered significant if $p \leq 0.05$. Results are presented as least-squares means (LSM) and their standard errors (SE).

3 Results

Physiological consequences of LPS administration. LPS induced an increase of TNF- α and IL-10 in blood plasma with maximum concentrations after 1 h ($p < 0.001$). Cortisol concentrations were increased 1, 3 and 6 h after LPS challenge ($p < 0.001$). The metabolites Kyn, Kyna, Quin were increased 6 h after LPS ($p < 0.01$). The ratio of Kyn/Trp as a marker for IDO activity was significantly increased after 3 h and reached maximum concentration after 6 h. (Fig. 1A). IDO activity did not differ from saline treated group 24 h after LPS

administration. However, in LPS treated pigs, the Trp level was significantly decreased from 3 up to 24 h after challenge compared to saline treated group ($p < 0.001$). The IFN- γ inducibility of blood cells by SEB stimulation was reduced 6 h ($p < 0.01$) and 24 h ($p < 0.1$) after LPS administration. Western blot analysis showed an influence of LPS on IDO protein expression in the liver and lung (Fig. 1B). IDO expression in muscle was not affected by the LPS treatment. IDO proteins with different molecular weight (34 kDa and 37 kDa) were detected in the liver whereas in lung only one form of IDO protein was found (34 kDa).

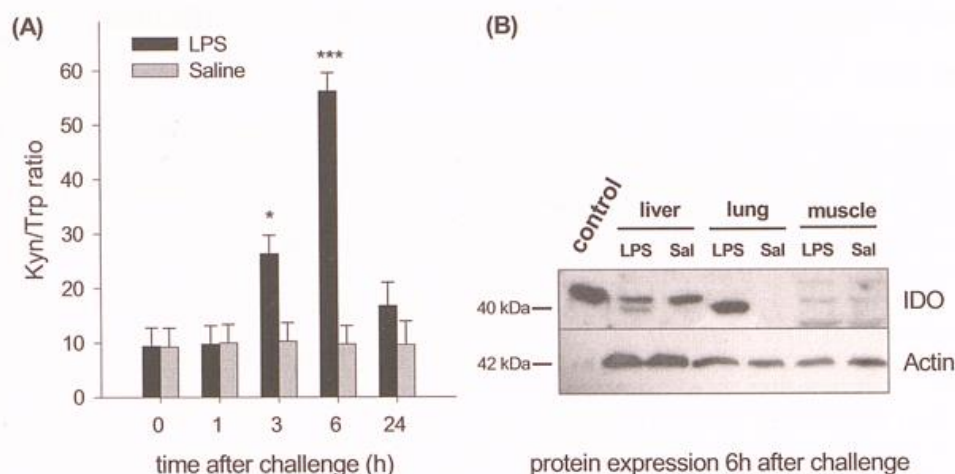


Fig. 1. Effects of *in vivo* LPS administration. (A) Kyn/Trp ratio in blood plasma ($n = 40$). (B) Expression of IDO protein in different tissues, 6 h after challenge ($n = 4$). * $p < 0.05$, *** $p < 0.001$

A model for blocking of IDO with 1-MT. After a single s.c. injection, the plasma level of 1-MT increased and reached a maximum level ($29.1 \pm 8.0 \mu\text{M}$) after approximately 12 h (Fig. 2A). Twenty four hours after injection, the plasma level of 1-MT was still elevated ($21.1 \pm 5.3 \mu\text{M}$). Repeating injections four times every 24 h generates an increased level of 1-MT followed by a saturated plasma level (Fig. 2B).

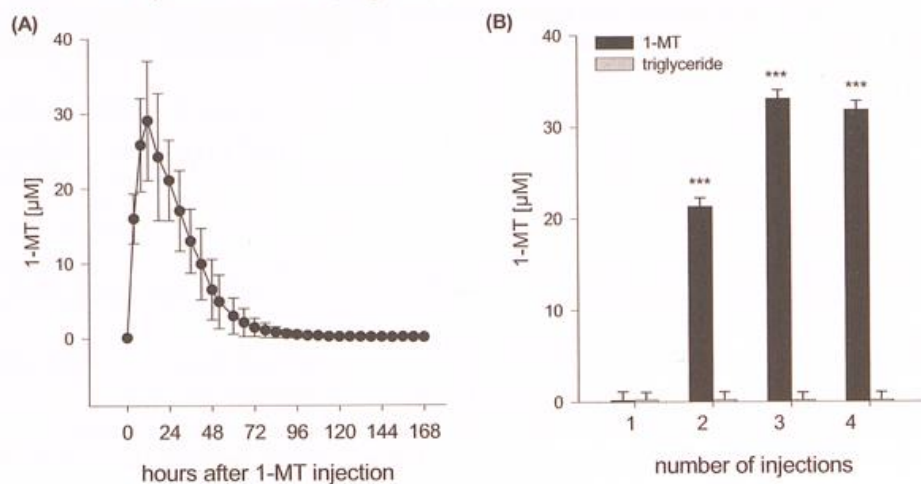


Fig. 2. Plasma level of 1-MT. (A) 1-MT after a single s.c. injection ($n = 3$). (B) 1-MT after repeated s.c. injections ($n = 47$). *** $p < 0.001$

4 Discussion

Our results show a depletion of Trp and accumulation of Kyn after LPS administration in pigs, which indicates an elevated enzymatic activity of IDO. These findings are supported by the western blot analysis detecting LPS induced IDO expression in the liver and in the lung. In addition, the existence of two detectable IDO forms in liver and blood cells (data not shown) supports the possibility of post-translational regulation of IDO activity (5, 6, 7). The increased plasma levels of TNF- α , IL-10 and cortisol show that LPS induces a pro- and anti-inflammatory immune response. Other studies confirmed that inflammatory stimuli such as TNF- α are able to stimulate IDO activity (2). IL-10 and cortisol are known to have immunosuppressive effects. The reduced reactivity of blood cells after *in vivo* LPS administration could be the consequence of this anti-inflammatory immune response. Also the depletion of Trp, caused by the increased IDO activity, is known to have antiproliferative effects in T-cells (3, 4). There are indications that IL-10 has suppressive effects on IDO inducibility (8) presuming that there is a well regulated balance between the activation of IDO by TNF- α and repressing by IL-10. Blocking of IDO with 1-MT *in vivo* provides new insights about the involvement of this enzyme in the immune response. Repeated subcutaneous injections increased the plasma concentration of 1-MT significantly, and a saturation of plasma 1-MT is achieved after two injections. This model is the basis for the current investigations determining the specific role of IDO after challenging with LPS.

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Social support alters behavioural and neuroendocrine responses to a single social isolation in juvenile pigs

Theresa Hameister, Birger Puppe, Margret Tuchscherer and Ellen Kanitz

Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany

1 Introduction

Psychosocial stress in the early neonatal phase of an individual plays an important role in the manifestation of short- and long-term effects on ethological and physiological mechanisms of adaptation, but is rarely examined in farm animals, especially in pigs (for review see [4]). Social isolation is a commonly used model for psychosocial stress and it was demonstrated that a single isolation of piglets led to changes in behaviour (e.g. enhanced locomotion and vocalisation) and physiological reactions (e.g. enhanced stress hormones, alterations in stress-related gene expression and immune response) [8-12]. In addition, there was also an age effect indicating that younger piglets are more vulnerable to this stressor. Interestingly, it was postulated already in the 1970s that social support by conspecifics could moderate such negative stress reactions [2]. However, the nature of the relationship between the individuals is crucial in determining whether or not social buffering will occur [6].

The present study was designed to examine the consequences of social support with familiar and unfamiliar conspecifics on behavioural responses and activity of the hypothalamus-pituitary-adrenal axis in socially isolated piglets. The effects of social partners were evaluated at three time points during suckling phase.

2 Materials and methods

A total of 324 German Landrace piglets were used in nine replicates. At 7, 21, or 35 days of age, four piglets from each litter were randomly allocated to an isolation treatment with or without social support (isolation with familiar conspecific (IF), isolation with unfamiliar conspecific (IU), isolation alone (IS)) or to a control group without any treatment (C). Piglets were isolated in a separate test room for 4 h in an opaque plastic box (0.68 x 0.50 x 0.65 m) and familiar, unfamiliar or none conspecifics were added. Within the box animals were separated by a transparent metallic fence. The control piglets remained undisturbed in the

farrowing pen during this time. Blood samples were taken from all piglets ($n = 18$ per treatment and age group) before and immediately after the treatment for hormone analysis. The differences of the stress hormone concentrations were generated by subtracting values before from the measure after treatment. After the second blood sampling some of the piglets were euthanized and their brains were prepared for qRT-PCR analysis of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) genes ($n = 9$ per treatment and age group). The behavioural responses of the piglets were tested in an open-field/novel-object test. Both before and after the treatment piglets were tested individually in a noise-reduced room in a 2.80×2.80 m open-field and the differences of the corresponding open-field behaviours were analysed (calculated by subtracting values before from the measure after treatment). Data were analysed by ANOVA using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The model of the investigated parameters contained the fixed effects replicate, treatment, age, sex, and interaction treatment $\times$ age. Least square means and their standard errors were calculated and tested for each effect in the model using the Tukey-Kramer procedure for all pairwise multiple comparisons. Significance was defined as $P < 0.05$.

3 Results

The repeated open-field/novel-object experience changed the behaviour of both control and experimental piglets towards a reduction of active behavioural patterns (vocalisation, locomotion, escape attempts). The changes in the active behavioural patterns were significantly affected by treatment (Table 1): duration of vocalisation ($P < 0.01$), duration of locomotion ($P < 0.01$) and duration of escape attempts ($P < 0.001$). Tukey-Kramer test revealed that, compared to controls, the decrease in escape attempts was significantly lower in piglets isolated alone and with unfamiliar conspecific, respectively. Additionally, both controls and piglets with familiar conspecific showed a greater reduction of locomotion compared to piglets isolated alone. Furthermore, the decrease of vocalisation was higher in piglets with familiar conspecific compared to animals isolated alone and with unfamiliar conspecific, respectively. No effect of treatment was found on duration of object contact ($P > 0.15$), but there was an effect on frequency of object contact ($P < 0.05$; Table 1).

Changes in the open-field/novel-object behaviour were significantly affected by age: duration of vocalisation ($P < 0.05$), duration of escape attempts ($P < 0.001$), duration and frequency of object contact ($P < 0.001$). Pairwise comparisons by Tukey-Kramer test revealed that piglets on day 7 displayed a lower reduction in the duration of vocalisation, escape attempts, and object contact and also lower reduction in frequency of object contact (data not shown).

Table 1: Changes in behavioural parameters measured in the open-field/novel-object tests. Data with different letters within a row differ significantly from each other (^{a,b} $P < 0.05$, Tukey-Kramer test).

Behaviour	Treatment ⁽¹⁾			
	C	IF	IU	IS
Vocalisation (sec)	-149.9 ± 15.5	-169.3 ± 15.8 ^a	-112.8 ± 15.7 ^b	-106.9 ± 15.9 ^b
Locomotion (sec)	-114.9 ± 12.4 ^a	-119.8 ± 12.1 ^a	-92.9 ± 12.2	-72.6 ± 12.2 ^b
Escape attempts (sec)	-21.4 ± 3.9 ^a	-9.6 ± 3.8	-0.6 ± 3.8 ^b	-2.2 ± 3.8 ^b
Object contact (sec)	-2.1 ± 4.2	-5.1 ± 4.1	3.6 ± 4.1	6.5 ± 4.1
Object contact (counts)	-2.7 ± 0.7	-3.1 ± 0.7	-1.0 ± 0.7	-0.9 ± 0.7

⁽¹⁾ C: control, IF: isolation with familiar conspecific, IU: isolation with unfamiliar conspecific, IS: isolation alone

The treatment significantly affected changes in the concentration of cortisol ($P < 0.001$) and the MR/GR mRNA ratio in prefrontal cortex ($P < 0.001$). As shown in Fig. 1A, piglets of all social treatments had significantly higher cortisol concentrations compared to controls. However, piglets with familiar ($P < 0.01$) and unfamiliar conspecific ($P = 0.1$), respectively, showed lower increases of cortisol concentrations than piglets isolated alone. Additionally, piglets isolated alone had a significant lower MR/GR mRNA ratio compared to controls and piglets isolated with an unfamiliar conspecific, respectively (Fig. 1B).

There was no age effect on cortisol changes ($P > 0.88$) and MR/GR mRNA ratio ($P > 0.74$; data not shown).

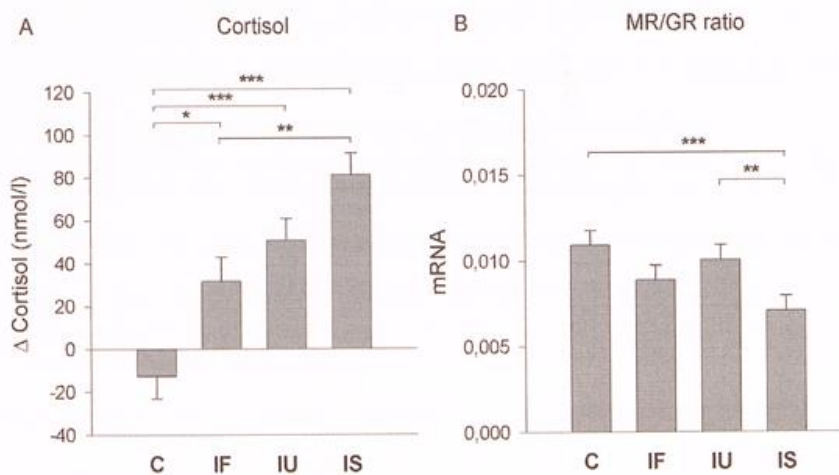


Fig. 1: Changes in plasma cortisol concentrations (A) and calculated MR/GR mRNA ratios in prefrontal cortex (B) in different treatment groups (C: control piglets, IF: isolation with familiar conspecific, IU: isolation with unfamiliar conspecific, IS: isolation alone). Significant differences (Tukey-Kramer test) between the groups are indicated by asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

4 Discussion

The present study demonstrates in a multidisciplinary approach that isolation with conspecifics alters behaviour, cortisol response and expression of MR and GR mRNA in a stress-related brain area of piglets. To our knowledge, this is the first complex investigation evaluating effects of social support on behaviour and neuroendocrine system in swine.

Findings in other mammals suggest that social partner may alleviate ethological and physiological responses to stressful situations [1; 5]. We found that isolation with social support attenuates behavioural reactivity in a repeated open-field test (e.g. reduction of vocalisation, locomotion, and escape attempts). Locomotion and vocalisation in an open-field test are known to be useful indicators for general activity and emotional arousal, respectively. Our results are in agree with a study of Herskin et al. [7], in which behavioural stress responses were also alleviated in partly isolated piglets, whereas fully isolated piglets perceived this treatment as a stressful condition. In our study, piglets isolated with a familiar conspecific were more passive in their behavioural responses than piglets isolated with an unfamiliar conspecific, indicating the crucial role of social relationship between individuals [6]. The lower increases of cortisol levels in socially supported piglets also emphasize the impact of social benefit. Furthermore, the imbalance of MR/GR-mediated effects in piglets isolated alone may indicate a dysregulation in control of stress, responsiveness, adaptation, and cognition [3], which was reversed by social support in the present study. The current results suggest that negative consequences of psychosocial stress could be buffered by social support probably leading to an enhanced ability to cope with new situations. The nature of the social relationship between the individuals plays also an important role. We conclude that, for reduction of psychosocial stress during weaning in livestock practice, the weaning age should be considered and it is beneficial when the weaned piglets are familiar with each other.

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Phosphorylierungsstatus von β -Catenin in bovinen Embryonen um den Zeitpunkt der Aktivierung des embryonalen Genoms

Heidi Leitner^{1,2}, Ralf Pöhland¹ und Wolfgang Tomek¹

¹Leibniz-Institut für Nutztierbiologie, Dummerstorf, ²Besamungsverein Neustadt a.d. Aisch e.V.

1 Einleitung

Während der präimplantatorischen Embryonalentwicklung kommt es zu einer Reihe von kritischen Ereignissen, deren korrekter Ablauf Voraussetzung für eine erfolgreiche Implantation ist. Dazu gehört die Aktivierung des embryonalen Genoms, die beim Rind im 8- bis 16-Zellstadium erfolgt (Telford et al., 1990) ebenso wie erste Differenzierungsvorgänge (Bell et al., 2008). Beide stehen in engem zeitlichen und kausalen Zusammenhang (Bhojwani et al., 2007; Watson und Barcroft, 2001).

In vorausgehenden Studien fand man Hinweise darauf, dass Störungen im Zytoskelett, insbesondere im Aktin-Catenin-Cadherin-System, eine reduzierte Differenzierungsfähigkeit und Entwicklungskompetenz von bovinen IVP-Embryonen mit verursachen können (Poehland et al., 2008). Zudem scheint β -Catenin auch im Zellkern dieser Embryonen eine Rolle zu spielen (unpublished data), was die Vermutung nahe legt, dass β -Catenin an der embryonalen Genomaktivierung beteiligt sein könnte.

β -Catenin ist ein multifunktionelles Protein, das in Zellmembran, Zytoplasma und Zellkern vorkommt. In der Zellmembran spielt es eine wichtige Rolle bei der Zelladhäsion (Jiang, 1996), im Zytoplasma wirkt es als Signalübertragungsmolekül und im Zellkern als Transkriptionskoaktivator (Willert und Nüsse, 1998). Der Funktionsstatus von β -Catenin wird durch Phosphorylierung reguliert, wobei dephosphoryliertes β -Catenin die aktive und phosphoryliertes β -Catenin die inaktive Form darstellt, die rasch proteasomal abgebaut werden kann (Aberle et al., 1997).

Vor dem Hintergrund einer eventuellen Beteiligung von β -Catenin an der Aktivierung des embryonalen Genoms sollte im Rahmen dieser Arbeit untersucht werden, wie sich β -Catenin hinsichtlich seines Phosphorylierungsstatus und Verteilungsmusters während der frühen präimplantatorischen Entwicklung boviner Embryonen, insbesondere während des kritischen 8-Zellstadiums, verhält.

2 Material und Methoden

Als Untersuchungsmaterial dienten bovine in vitro produzierte Embryonen vom 4- bis zum 16-Zellstadium. Die für die Gewinnung der Embryonen benötigten Oozyten wurden mittels Follikelaspiration aus Ovarien von Schlachtkühen der Rasse Deutsches Fleckvieh gewonnen und nach gängigem IVP-Protokoll gereift, befruchtet und kultiviert. Im ersten Versuchsabschnitt sollte untersucht werden, ob und gegebenenfalls wann es im Verlauf der Entwicklung vom 4- zum 16-Zellstadium zu nachweisbaren Veränderungen im Phosphorylierungsstatus und somit in der Aktivität von β -Catenin kommt. Hierzu wurde mittels Westernblot das Level an Gesamt- und Phospho- β -Catenin in den unterschiedlichen Entwicklungsstadien bestimmt.

Um nähere Aussagen zu der Funktion von β -Catenin im Embryo machen zu können, wurde mit Hilfe der konfokalen Laserscanningmikroskopie und anschließender Bildanalyse das Verteilungsmuster von Gesamt- und Phospho- β -Catenin in unterschiedlichen Entwicklungsstadien untersucht.

In zwei weiteren Experimenten wurde analysiert, wie sich der Einsatz eines GSK3-Inhibitors in unterschiedlichen Konzentrationen auf den Phosphorylierungsstatus (Westernblot) und das Verteilungsmuster (LSM) von β -Catenin auswirkt. GSK3 ist ein Enzym, das unter anderem für die Phosphorylierung von zytoplasmatischem β -Catenin zuständig ist und dadurch die Voraussetzung für dessen proteasomale Degradierung schafft (Aberle et al., 1997).

Um das Translokationsverhalten von β -Catenin innerhalb des Embryos analysieren zu können, wurde eine Überexpression von YFP-markiertem β -Catenin durch Injektion eines entsprechenden Plasmides erzeugt. Die YFP-Lokalisation wurde während der Entwicklung vom 4- zum 16-Zell-Embryo mittels Live Cell Imaging kontinuierlich beobachtet. FRAP-Analysen dienten zum Nachweis des zeitlichen Verlaufs der Translokationsprozesse.

Bei einem P-Wert $< 0,05$ wurden ermittelte Unterschiede als statistisch signifikant betrachtet.

3 Ergebnisse

Im Westernblot ergaben sich weder für Gesamt- noch für Phospho- β -Catenin quantitative Unterschiede zwischen den untersuchten Entwicklungsstadien. Der Anteil von Phospho- β -Catenin an Gesamt- β -Catenin war durchgehend extrem niedrig, allerdings trat im niedermolekularen Bereich eine weitere, sehr starke Bande auf, bei der es sich vermutlich um ein die Phosphorylierungsstelle enthaltendes Spaltstück von β -Catenin handelt.

Der eingesetzte Inhibitor zeigte keinerlei Auswirkungen auf die Proteinlevels von Gesamt- oder Phospho- β -Catenin.

Bezüglich des Verteilungsmusters von Gesamt- β -Catenin waren ebenfalls kaum Unterschiede zwischen den einzelnen Entwicklungsstadien zu erkennen. Gesamt- β -Catenin fand sich überwiegend membranständig, zu einem wesentlich geringeren Anteil im Zytoplasma und nur in geringen Spuren im Zellkern. Auffällig war eine im Laufe der Embryonalentwicklung zunehmende perinukleäre Verdichtung von zytoplasmatischem β -Catenin.

Während Phospho- β -Catenin in Zellmembran und Zytoplasma ein im Verlauf der Entwicklung von 4- zum 16-Zellstadium konstant bleibendes Verteilungsmuster aufwies, wurden im Zellkern signifikante Unterschiede zwischen den einzelnen Entwicklungsstadien deutlich: Vom 4- bis hin zum 16-Zellstadium kam es zu einem stetigen Anstieg der Fluoreszenzintensität von Phospho- β -Catenin, wobei die Werte im 4- und 6-Zellstadium noch signifikant unter und ab dem späten 8-Zellstadium dann signifikant über denen in Zellmembran und Zytoplasma lagen. Parallel hierzu kam es innerhalb des Zellkerns zu einer Verschiebung der Lokalisation von Phospho- β -Catenin von einem zunächst diffusen zu einem zunehmend zonalen Verteilungsmuster.

Der Einsatz des GSK3-Inhibitors in seiner höchsten Konzentrationsstufe (500 nM) führte zu einer je nach Entwicklungsstadium teils signifikanten Verringerung der perinukleären Verdichtung von Gesamt- β -Catenin. Im Falle von Phospho- β -Catenin zeigte sich ebenfalls eine je nach Entwicklungsstadium teils signifikante Verringerung der Fluoreszenzintensität im Zellkern, begleitet von einem ab dem späten 8-Zellstadium signifikanten Absinken des Anteils von Embryonen mit zonalem Verteilungsmuster.

Die FRAP-Analysen ergaben beim Bleichen des gesamten Embryos eine 1,5tägige Recovery-Dauer, während beim alleinigen Bleichen des Zellkerns die Färbung bereits nach 30 Minuten wiederhergestellt war.

4 Diskussion

In bovinen Präimplantationsembryonen unterschiedlicher Entwicklungsstadien ist ein konstant bleibendes Level an Phospho- β -Catenin bzw. dessen Spaltprodukt nachweisbar. Da sich dieses Level trotz Hemmung der de novo Phosphorylierung durch den eingesetzten GSK3-Inhibitor nicht verringert, ist davon auszugehen, dass dieses Produkt während der Entwicklung vom 4- zum 16-Zellembryo entgegen des üblichen Weges der proteasomalen Degradierung nicht weiter abgebaut wird. Vielmehr kommt es, wie im LSM ersichtlich, im Verlauf der Embryonalentwicklung zu dessen Anreicherung im Zellkern. Aus den FRAP-Analysen wird ersichtlich, dass diese Anreicherung auf einer aktiven Translokation von phosphoryliertem β -Catenin bzw. eines die Phosphorylierungsstelle enthaltenden

Spaltproduktes von β -Catenin aus dem Zytoplasma in den Zellkern beruht. Die im LSM beobachtete perinukleäre Verdichtung von β -Catenin könnte hiermit in Zusammenhang stehen. Nach bisherigen Erkenntnissen spielt dephosphoryliertes β -Catenin im Zellkern im Rahmen der Wnt-Signalübertragung eine wichtige Rolle für die Transkriptionsaktivierung (Staal et al., 2002), allerdings ist der Wnt-Signalweg bei frühen Präimplantationsembryonen vermutlich noch gar nicht vollständig aktiv (Harwood et al., 2008). Vielmehr scheint hier phosphoryliertes β -Catenin bzw. dessen Spaltstück im Zellkern eine physiologische Funktion zu übernehmen. Um welche Funktion es sich hierbei konkret handelt, ist noch nicht bekannt.

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Vergleichende Untersuchungen zu verschiedenen Reproduktionsbiologika beim Schwein

Bernd Kruse und Klaus-Peter Brüssow

Leibniz-Institut für Nutztierbiologie Dummerstorf, FB Fortpflanzungsbiologie

1 Einleitung

Biotechnische Verfahren zur Steuerung des Sexualzyklus erfahren in effizient wirtschaftenden Ferkelerzeugerbetrieben eine zunehmende Bedeutung, um durch Gruppenabferkelsysteme den ökonomischen und arbeitstechnischen Forderungen gerecht werden zu können. Allgemein ist Biotechnik als die Steuerung und Nutzung biologischer Systeme durch den Menschen zur gezielten Produktion von Stoffen zu verstehen. Die spezielle Biotechnik der Fortpflanzung verfolgt durch Beeinflussung des weiblichen Reproduktionsapparates das Ziel, die physiologischen Prozesse von Follikelwachstum, Follikelreifung, sowie Ovulation und Geburt zeitgleich in Sauengruppen auszulösen (BRÜSSOW und WÄHNER 2005).

Durch Injektion von Stutenserumgonadotropin (pregnant mare serum gonadotropin, PMSG, equines Choriongonadotropin, eCG), welches im Endometrium der Stute zwischen dem 35. und 80. Graviditätstag in hohen Konzentrationen und bis zum 120. Trächtigkeitstag in fallenden Konzentrationen gebildet wird (COLE und GOSS 1943, BERGFELD et al. 1978), kann bei Alt- und Jungsauen das Follikelwachstum angeregt werden. Die Wirkung des PMSG ist dabei sowohl Follikel stimulierend als auch luteinisierend (FARMER und PAPKOFF 1979).

Seit 2008 ist zudem das Produkt Maprelin® XP10 der Firma Veyx Pharma GmbH für die Brunststimulation bei Jung- und Altsauen zugelassen. Bei diesem Präparat handelt es sich um ein synthetisches Lamprey-GnRH-III (IGnRH-III; Wirkstoffname Peforelin), dem eine selektive FSH-Ausschüttung zugeschrieben wird. Während in Untersuchungen an Börgen mit 150 µg IGnRH-III eine FSH-Ausschüttung induziert wurde (KAUFFOLD et al. 2005), konnte bei zyklischen Jungsauen jedoch keine FSH-Sekretion nach IGnRH-III-Applikation nachgewiesen werden (BRÜSSOW et al. 2010).

Ziel dieser Studie war es in Praxisexperimenten und ultrasonographischen Untersuchungen zu prüfen, inwieweit Peforelin eine vergleichsweise zum PMSG gleichwertige Stimulation von Follikelwachstum und Brunst bewirkt und welche Fruchtbarkeitsleistungen nach Applikation dieser Reproduktionsbiologika erreicht werden.

2 Material und Methoden

Insgesamt wurden 512 Jungsauen, 236 primipare und 743 multipare Sauen in jeweils drei Gruppen (Peforelin, PMSG und Kontrolle) unterteilt und vergleichend Fruchtbarkeitsparameter wie Östrusrate, Trächtigkeits- und Abferkelergebnisse erhoben.

Die Jungsauen wurden 48 Stunden nach einer 18-tägigen Zyklussynchronisation mit Regumate® (Altrenogest) mit Maprelin® XP10 (Peforelin) oder Pregmagon® (PMSG) behandelt, primiparen und multiparen Sauen wurden hingegen 24 Stunden nach dem Absetzen mit einem der beiden Wirkstoffe behandelt. Zusätzlich wurde ein Teil der Versuchstiere durch ultrasonographische Ovaruntersuchungen auf Follikelwachstum und Ovulationszeitpunkt untersucht. Es wurde die Wirkung der Reproduktionsbiologika auf Östruseintritt und -dauer geprüft und morgens und abends von Tag 4 bis Tag 8 nach dem Absetzen ein Ovarstatus erhoben. Die Untersuchungen erfolgten transkutan mit dem tragbaren Ultraschallgerät HS 101V, welches mit einem 5 MHz Microconvex-Schallkopf und einem 5,7 LCD Monitor ausgestattet war.

Die statistischen Analysen wurden mit der SAS/STAT Software, Version 9.2 des SAS Systems für Windows, Copyright © 2009 SAS Institute Inc., durchgeführt.

Für relative Häufigkeiten als Zielvariable wurde ein logistisches Modell verwendet (Setzen der Optionen: DISTRIBUTION=BIN, LINK=LOGIT). Zählvariablen wurden mittels des Poisson-Modells (DISTRIBUTION=POISSON, LINK=LOG) und kontinuierliche Variablen mittels eines Varianzanalysemodells (Setzen der Optionen: DISTRIBUTION=NORMAL, LINK=IDENTITY) analysiert.

Die multiplen Mittelwertvergleiche erfolgten mit dem Tukey-Kramer-Test, was durch Setzen der Option ADJUST=TUKEY im LSMEANS-Statement der GLIMMIX-Prozedur realisiert wurde.

3 Ergebnisse

Die Östrusrate der PMSG-behandelten Sauen unterschied sich in allen Altersgruppen (JS, PS, AS) deutlich von den Peforelin-behandelten Sauen und den Kontrolltieren. Altersklassen übergreifend konnten die PMSG-behandelten Sauen (89,0%) signifikant höhere Östrusraten als die Peforelin- bzw. Kontrolltiere (76,7 bzw. 78,1%) erzielen (Abb. 1). Das Brunststimulations-Beleg-Intervall war Altersklassen übergreifend signifikant verkürzt zwischen der PMSG-Gruppe (3,98 Tage) im Vergleich zur Peforelin- und Kontrollgruppe (4,34 bzw. 4,48 Tage) (Abb. 2).

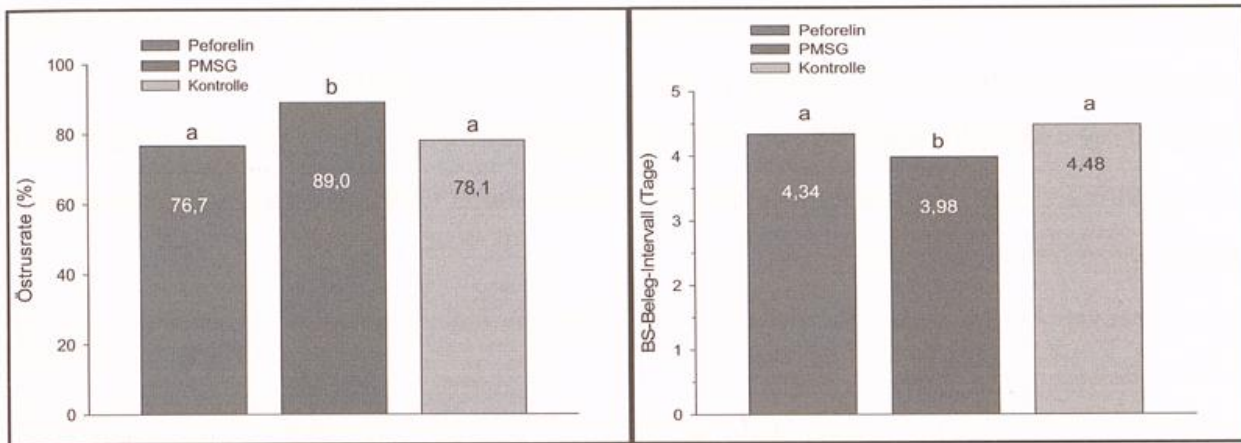


Abb. 1: Östrusrate, Altersklassen übergreifende Gruppendarstellung
a, b ($p < 0,05$)

Abb. 2: Brunststimulations-Beleg Intervall aller Altersklassen
a, b ($p < 0,05$)

Während die Trächtigkeits- und Abferkelraten der multiparen Sauen unabhängig von den Behandlungsgruppen vergleichbar hoch waren, konnten sowohl bei den Jungsaugen als auch bei den primiparen Sauen die Kontrollgruppen die besten Ergebnisse erzielen und lagen oberhalb der PMSG- und Peforelingruppen. Die Wurfgrößen der Jungsaugen, primiparen und multiparen Sauen waren in allen drei Behandlungsgruppen vergleichbar.

Die ultrasonographischen Untersuchungen der multiparen Sauen ergaben ein Absetz-Östrus-Intervall der PMSG-Gruppe von 90,7 h im Vergleich zur Peforelin-Gruppe mit 100,3 h und zur Kontrollgruppe mit 95,6 h. Auch das Absetz-Ovulations-Intervall war in der PMSG-Gruppe mit durchschnittlich 135,1 h geringer als in der Peforelin- und Kontrollgruppe (146,3 bzw. 144,6 h). Die mit PMSG-behandelten Sauen ovulierten 11,2 h vor den Peforelin-behandelten Sauen und 9,5 h vor den Sauen aus der Kontrollgruppe.

4 Diskussion

Die Ergebnisse zeigen, dass der Wirkstoff Peforelin in der hier verwendeten Dosierung keine Stimulation von Follikelwachstum und Brunst bewirkt. Peforelin vermochte weder die Östrusrate zu steigern, noch eine Verkürzung der Leertage nach dem Absetzen bzw. nach Regumategabe zu bewirken. Obwohl biochemische Untersuchungen ergeben haben, dass lGnRH-III eine 60%ige Homologie zu GnRH-I hat (WATTS et al. 2001), wird eine ausreichende Bindung an den GnRH-Rezeptor beim Schwein wahrscheinlich nicht erreicht. lGnRH-III bindet selektiv nur an den lGnRH-III-Rezeptor (SILVER und SOWER 2006) und eine Bindung an den GnRH-I-Rezeptor wurde bisher nicht nachgewiesen. Das kann durch die unterschiedliche Struktur des GnRH-I-Rezeptors im Vergleich zu den GnRH-II- und -III-Rezeptoren bedingt sein (NOAR et al. 2005, SCHNEIDER et al. 2006). Darüber hinaus

wurde lGnRH-III und sein Rezeptor bisher nicht bei Säugetieren, das Schwein eingeschlossen, isoliert (MORGAN et al. 2006, MILLAR et al. 2008).

Anhand der vorliegenden Arbeit kann bestätigt werden, dass sich die transkutane Ultraschalluntersuchung zur Erfassung des Ovarstatus sowie zur Bestimmung des Ovulationszeitpunktes eignet.

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Comparative analyses of apoptosis-related candidate genes in rainbow trout: molecular characterization and transcriptome analyses

Marieke Verleih¹, Alexander Rebl¹, Bernd Köllner², Tomáš Korytář², Carsten Kühn³, Klaus Wimmers¹ and Tom Goldammer¹

¹ Leibniz-Institut für Nutztierbiologie (FBN), Fachbereich Molekularbiologie, Dummerstorf, Germany

² Friedrich-Loeffler-Institut (FLI), Institut für Infektionsmedizin, Greifswald, Insel Riems, Germany

³ Landesforschungsanstalt für Landwirtschaft und Fischerei Mecklenburg-Vorpommern (LFA-MV), Institut für Fischerei, BORN, Germany

1 Introduction

Programmed cell death, known as apoptosis [1], facilitates the active elimination of damaged, mutated or infected tissue [2] and therefore promotes the maintenance of normal tissue functions. The molecular mechanisms of the mammalian apoptosis pathway are comprehensively examined and evolutionary well conserved [3;4]. Cysteine-aspartic proteases, termed as caspases represent the central protein family involved in programmed cell death. Apoptosis is initiated by extracellular death ligands, e.g. the cytokines FASLG (Fas ligand) and TNF α (tumor necrosis factor- α) that bind to their specific membrane-bound death receptors. The following intracellular signalling cascade activates the caspases, which in turn regulate the final apoptotic steps. Beside this extrinsic pathway, an intrinsic cascade is known, which activates the caspases by the release of CYCS (cytochrome c, somatic) and DIABLO (direct IAP-binding protein with low pI).

The use of fish as a model organism for apoptosis studies has been recently recommended since teleosts can be studied under extreme environmental conditions that cannot be easily investigated in mammals, e.g. fluctuations of temperature, salinity or oxygen content [5]. Several crucial molecules of the programmed cell death in commercially important rainbow trout have been characterized except for initial CASP3 (caspase-3) and effector CASP8 (caspase-8) gene. In parallel to the structural characterization of those factors, we compared the strain-specific expression profiles of apoptosis-related genes *via* transcriptome analyses in two aquacultured rainbow trout (*Oncorhynchus mykiss*) lines after a pathogen infection: the local selection strain BORN (Germany) and the imported strain TCO from Tacoma (USA). Compared to TCO strain, the BORN strain shows an increased resistance towards different stress factors such as infection and pollution [6]. A different regulation of several molecular

factors, putatively involved in the defense of stress and infection has been described recently [7;8]. Therefore, both lines serve as a suitable model organism to study varying adaptation to environmental stress.

2 Materials and methods

Eleven to twelve month old BORN and TCO rainbow trout, grown under identical conditions in a regional aquaculture farm (BIMES, Frauenmark, Germany) for nine month and then transferred and adapted to glass tanks for further three months, were infected intraperitoneally (i.p.) with a dosage of 1×10^4 cfu (colony forming unit) of the pathogen *Aeromonas salmonicida* subsp. *salmonicida*. Trunk kidney tissue samples of four fish per strain were taken 0 h (control) and 72 h as well as 7 d and 21 d after infection, transferred into RNeasy lysis buffer, and subsequently homogenized in 1 ml Trizol (Invitrogen, Karlsruhe, Germany). RNA extraction was conducted using RNeasy Mini Kit (Qiagen, Hilden, Germany) with in-column DNase treatment for 30 min or DNA extraction by using QIAamp DNA Micro Kit (Qiagen). The integrity of RNA was verified by agarose gel electrophoresis.

Subsequently, total RNA of trunk kidney tissue was hybridized to a 4x44K Agilent salmonid oligo microarray according to a standard protocol by ATLAS Biolabs GmbH (Berlin) [9]. For each time-point, samples from two animals per strain were included in hybridization experiments. Data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

3 Results and discussion

We compared the temporal expression of 47 apoptosis-related genes present on the salmonid microarray chip in both rainbow trout lines BORN and TCO after infection with the intracellular gram-negative bacterium *A. salmonicida* subsp. *salmonicida*. The expression profile in trunk kidney revealed significant differences within both strains, in particular 7 days after infection compared to uninfected fish (Fig.1).

Both strains show no significant regulation of apoptosis-related genes 72 hours after infection in comparison to uninfected control fish except for TANK (TRAF family member-associated NF- κ B activator) which is up-regulated in BORN trout (1.71-fold, $p = 0.006$) and responsible for blocking the NF- κ B (nuclear factor NF-kappa-B) activation. In contrast, significant regulation of in total ten apoptosis-related genes could be detected 7 d after infection. In BORN rainbow trout, (i) the anti-apoptotic gene BCL-2 (B-cell CLL/lymphoma 2) is -4.03-

fold ($p=0.003$) down-regulated and (ii) the pro-apoptotic effector CASP 9 (caspase-9) gene is 1.90-fold ($p=0.04$) enhanced.

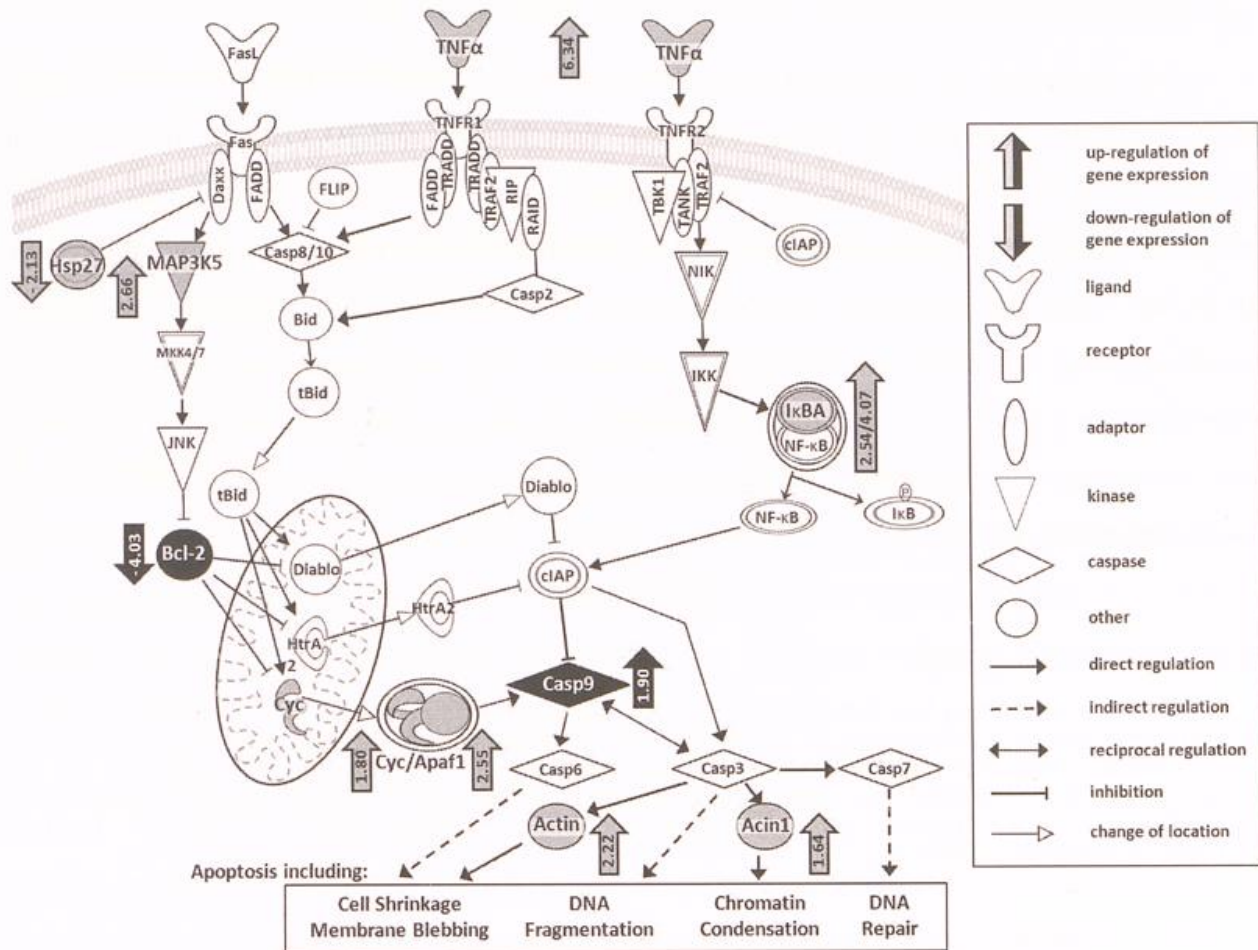


Fig.1: Regulation of apoptosis-related genes in trunk kidney after pathogen infection. Transcript level 7 d after infection was compared to uninfected rainbow trout (0 h). Significantly ($p < 0.05$) regulated genes of BORN and TCO trout are represented by black and grey symbols, respectively; gene-specific fold-change is indicated in black and grey arrows, respectively.

Eight apoptosis-related genes are significantly regulated in TCO trout compared to healthy trout. The cell death-inducing death-receptor ligand $TNF\alpha$ is elevated 6.34 fold ($p=0.01$) as well as the pro-apoptotic genes MAP3K5 (mitogen-activated protein kinase kinase kinase 5; 2.66-fold, $p=0.025$), CYCS (1.80-fold, $p=0.037$) and APAF1 (apoptotic peptidase activating factor 1; 2.6-fold, $p=0.045$). Additional, gene expression of HSP27 (heat shock protein 27) inhibiting Fas-mediated apoptosis, is decreased -2.13-fold ($p=0.009$), whereas the transcript levels of two putative NF- κ B inhibitor gene variants for NFKBIA (nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha) are elevated 2.54/4.07-fold ($p=0.03/0.009$). Furthermore, we detected a significant up-regulation of ACTA2 (smooth muscle aortic alpha-actin; 2.22-fold, $p=0.011$) and ACIN1 (apoptotic chromatin condensation inducer in the nucleus; 1.64-fold, $p=0.009$), which are both involved in the final steps of apoptosis, leading to cell shrinkage, membrane blebbing and chromatin condensation. Three

weeks after infection, no significant gene regulation could be observed in BORN trout. However, effector caspase 3 transcript level was elevated 1.69-fold ($p=0.006$) in TCO trout at this time point.

In summary, we detected a pro-apoptotic gene regulation in both rainbow trout lines after pathogen infection. It seems, however, that BORN and TCO trout use different distinctive regulation mechanisms. Interestingly, in TCO trout, eight genes of the programmed cell death were regulated, located at different steps of three apoptotic pathways. In BORN trout, the regulation was restricted to two genes of the Fas-mediated apoptosis. Verification of the results *via* qRT-PCR is underway. The data support and consolidate our hypothesis of a genetically determined optimized adaptation of BORN trout to local habitats and environmental conditions. This strain is therefore suggested as a valuable alternative in local aquaculture farms.

4 Acknowledgements

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Transcriptome Analysis of Porcine PBMC after Vaccination with Tetanus Toxoid as a Model Antigen

Marcel Adler, Eduard Murani, Ronald Brunner, Siriluck Ponsuksili and Klaus Wimmers

Leibniz Institute for Farm Animal Biology (FBN), Research Unit Molecular Biology, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

1 Introduction

In animal husbandry, the expression of traits of interest is often subject to the effects of multiple genes. Beside additive effects, several kinds of gene interaction cause non additive antagonistic or synergistic effects. Considering the resulting variation of complex traits, different kinds of interaction can be observed between functional and performance traits.

In pig farming, the incidence and severeness of infectious diseases has direct influence on animal welfare, product quality and economics. So the investigation of underlying immunogenetic fundamentals plays a major role. Sustaining the pig's genetic resistance to infection has been considered as a key breeding goal to improve disease prophylaxis (Reiner 2009).

Little is known about the genetic control within the relationship between functional traits and performance traits. In poultry, the selection for high growth rates resulted adversely in an impaired immune competence (Swaggerty et al. 2009). However, pigs selected for high immune response showed also an enhanced weight gain (Wilkie and Mallard 1999).

This study examines the relationship between performance traits and functional traits of the immune system. Other studies of transcriptional regulation during the immune response in peripheral blood mononuclear cells (PBMC) so far were limited to candidate genes (Choi et al. 2002, Suradhat et al. 2003) or performed *in vitro* with immune specific non-genomic microarrays (Gao et al. 2010). Here, to get a more comprehensive insight into gene regulation within the immune response, weaning pigs were vaccinated using tetanus toxoid as a model antigen for a TH1 and TH2 immune response and expression profiles of PBMCs before and several time points after immunisation are displayed via genome-wide microarrays (Ponsuksili et al. 2008).

2 Material and methods

After weaning animals (n=223) of a German Landrace herd were vaccinated with tetanus toxoid. Directly before (0), as well as 2, 4, 8, 30 and 75 hours after vaccination blood samples were collected. Vaccination and blood sampling were replicated 14 days after the first vaccination, a final blood sample was taken at day 28. From 4 ml of blood PBMCs were isolated by centrifugation on a Histopaque (Sigma) density gradient. Complete blood counts and anti-tetanus toxoid antibody titers were obtained and the animals were performance tested.

In order to get a first insight into the transcriptional response of PBMCs to the vaccination a subset of three male and three female piglets from three litters respectively (n=18) were analysed. For the hybridisation on Affymetrix GeneChip Porcine Genome Arrays three RNA pools were generated each of six individuals, one male and one female from each litter.

The microarray raw data was processed using the algorithms MAS5.0 and PLIER. Pairwise comparisons of each timepoint after vaccination against 0 hours were subjected to a variance filter and a paired t-test. Beside these analyses of single timepoints, a time course analysis was carried out via K-Means Clustering by MeV software (Saeed et al. 2003). The bioinformatic analysis of significantly regulated transcripts ($p < 0.05$) was carried out by use of a recent annotation of Affymetrix probe sets (Naraballobh et al. 2010) and the Ingenuity Pathways Analysis (IPA) Software (<http://www.ingenuity.com/>).

3 Results and discussion

The genomic microarray analysis of RNA from porcine PBMC after *in vivo* immune stimulation by tetanus toxoid shows in total > 5000 regulated genes ($p < 0.05$). Within the single time stages the number of differentially expressed genes is between several hundred and more than 1000 genes with different proportions of up and down regulation (table 1). Our bioinformatic analyses based on the IPA knowledge base reveal differentially expressed genes from canonical pathways concerning the immune response as well as several functions in cell organisation, metabolism and biosynthesis (Figure 1).

The results show that significant changes in the expression profiles of PBMC occurred very early after *in vivo* immune stimulation. In terms of immune function, regulations are present for Interleukin, Interferon-, NK-, B cell and T cell specific pathways.

Further regulation of pathways, like Integrin, JAK/Stat, Growth Factor, Protein Ubiquitination and ERK/MAPK signaling, controls processes in cell cycle, communication, proliferation and development. In overlap with these, processes like stress response and apoptosis are found represented by SAP/JNK and Glucocorticoid metabolism.

Table 1: Number, direction and FDR values of significantly regulated genes ($p < 0.05$) over the timepoints of blood sampling after tetanus vaccination

Time after 1st vaccination	2 h	4 h	8 h	30 h	75 h	d 14
Number of regulated genes	679	1196	1104	773	549	642
Up regulation	417	1063	889	233	118	207
Down regulation	262	133	215	540	431	435
FDR (at $p = 0.05$)	0.24	0.08	0.08	0.22	0.36	0.21
Time after 2nd vaccination	2 h	4 h	8 h	30 h	75 h	d 28
Number of regulated genes	485	358	824	942	1121	527
Up regulation	301	187	406	557	259	408
Down regulation	184	171	418	385	862	119
FDR (at $p = 0.05$)	0.49	0.59	0.24	0.15	0.09	0.32

These data indicate a co-regulation of immune system, metabolism and endocrine system. A comparison of PMBC expression profiles among individuals with divergent immune responsiveness and divergent performance will enable the identification of key genes in regulatory and metabolic pathways. In addition, the impact of genetic variability and the mode of interaction and coordinated regulation of these genes will be studied. Therefore, regulatory networks, epistatic and pleiotropic effects, and linkage disequilibrium among key genes for functional and production traits will be addressed. The results will provide insights into the genetic control of phenotype expression within the relationship of functional and performance traits.

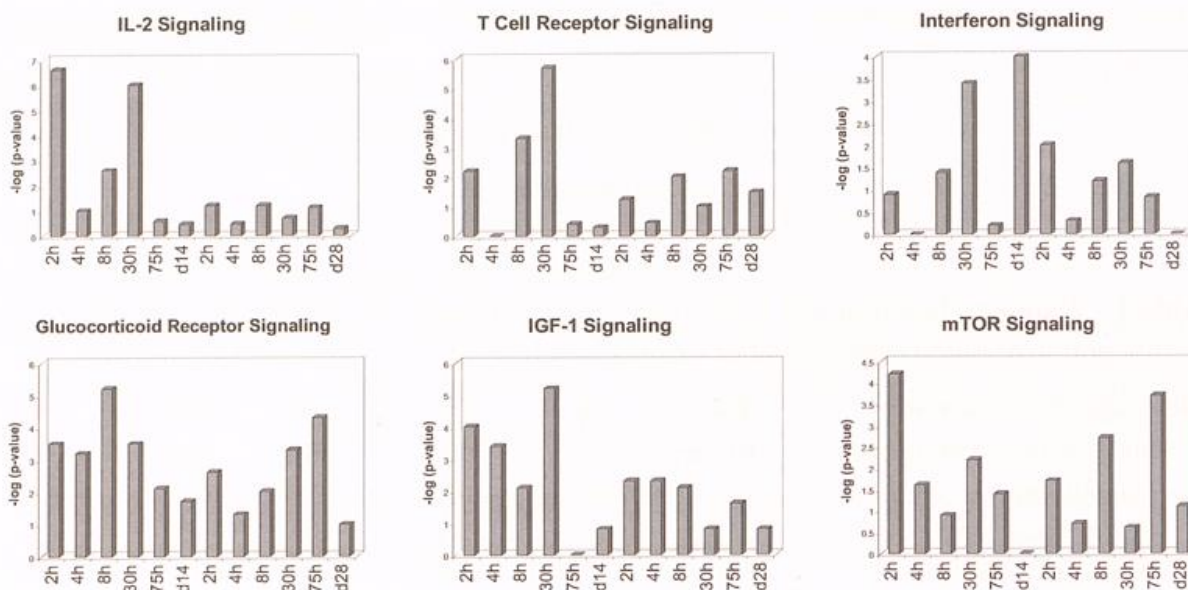


Figure 1. A selection of regulated canonical pathways of immune specific and other cell biological functions; the significance of regulation within the pathways is presented by bars.

4 Acknowledgements

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Phosphatase und Tensin Homolog (PTEN) als neuer Biomarkerkandidat für postnatalen Muskelansatz in der Maus

Mandy Sawitzky¹, Peggy Stock², Martina Langhammer¹, Ulla Renne¹ und Andreas Höflich¹

¹Genetik & Biometrie, AG Mausgenetik, Leibniz-Institut für Nutztierbiologie (FBN), Dummerstorf,

²ZAMED, Molekulare Hepatologie, Martin-Luther Universität, Halle

1 Einleitung

Zahlreiche Arbeiten belegen eine zentrale Rolle der Proteinkinase B (AKT) für den Muskelansatz in Säugetieren (Bodine et al). Auch wir konnten bei der Langzeitanalyse der Dummerstorfer Mauslinie DU6P (Selektion auf hohe Proteinmenge, Bünger et al) in erwachsenen weiblichen Mäusen eine massive AKT Aktivierung feststellen. Wir haben daher unser Tiermodell verwendet, um die Mechanismen zu untersuchen, die an der Kontrolle von AKT beteiligt sind. In unsere Untersuchungen wurden die möglichen Funktionen des Insulin-ähnlichen Wachstumsfaktor (IGF)-Systems, der Integrin-linked Kinase (ILK), von Myostatin (GDF-8) und PTEN mit einbezogen. Wir untersuchten weiter die Folgen der erhöhten AKT Aktivierung auf die Kontrolle und Ausprägung des Glykogen- und Proteinstoffwechsels in erwachsenen weiblichen DU6P Mäusen. Dabei ließen sich spezifische Aktivierungskaskaden schlüssig bis zum Glykogen- und Proteingehalt im Muskel darstellen. Die Ergebnisse der Studie lassen eine Schlüsselrolle von PTEN bei der Kontrolle des Glykogen- und Proteinstoffwechsels in adulten weiblichen Mäusen vermuten und weisen in unserem Auszuchtmodell PTEN als funktionalen Biomarker für Muskelansatz aus.

2 Material und Methoden

In einer Langzeitstudie wurden die Massen des isolierten *Musculus quadriceps femoris*, der Karkasse sowie des gesamten Körpers von Mäusen beider Geschlechter der unselektierten Kontrolle Fzt:DU (142. Generation) und der Proteinlinie DU6P (138. Generation) erfasst (n = 15). Aus *M. quadriceps femoris* wurde die Proteinfraction isoliert und verschiedene Signalmoleküle mittels Western-Immunoblot bestimmt (n = 3-9). Für den Schwerpunkt der Analysen wurden 3 Zeitpunkte (11, 29, 54 Wochen) ausgewählt. Die Muskeln von weiblichen 29 Wochen alten Tieren wurden hinsichtlich Glykogen chemisch und histologisch analysiert. Die statistische Auswertung erfolgte mit ANOVA der JMP 8 Software. Die Grafiken zeigen Mittelwerte $\pm$ SEM. Statistische Signifikanz wurde bei $p < 0,05$ angenommen.

3 Ergebnisse und Diskussion

Mit der Selektion auf hohe Proteinmenge in der Linie DU6P von ursprünglich ca. 3 g in den ersten Generationen auf 6,7 g in der 138. Generation ist neben einem signifikant erhöhten Körpergewicht auch ein höheres Muskel- und Karkassengewicht korreliert (Abbildung 1).

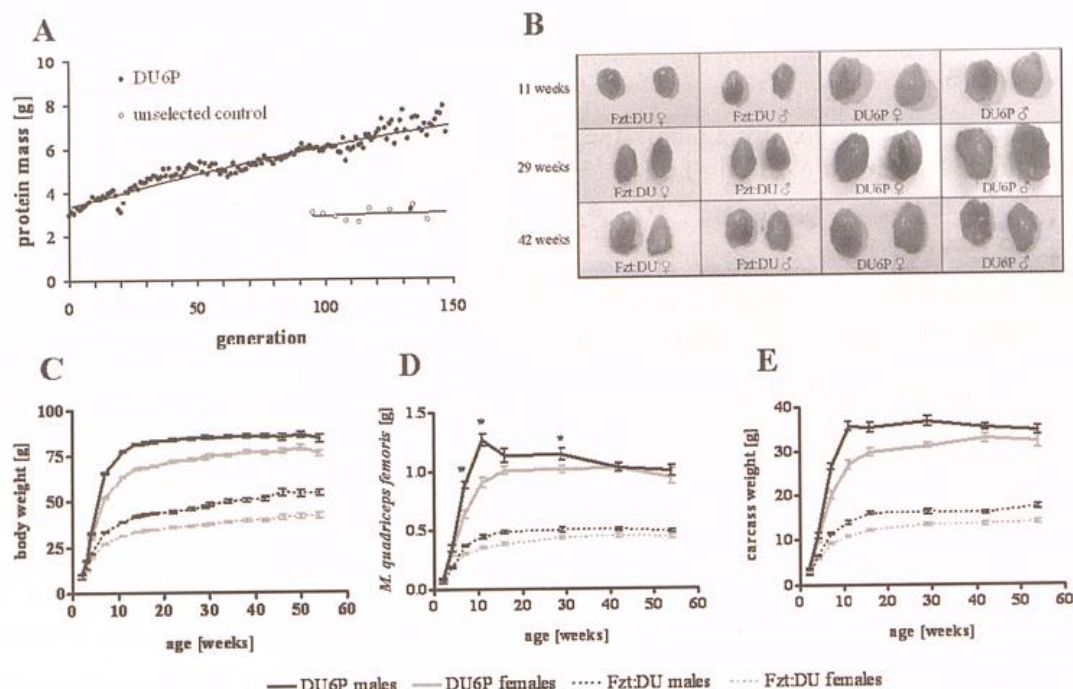


Abbildung 1: Phänotyp von DU6P Mäusen vs. unselektierte Kontrollen (Fzt:DU). A: Steigende Proteinmenge der Linie DU6P während der Selektion. B: Präparierter *M. quadriceps femoris* von 11, 29 und 42 Tage alten Mäusen beider Mauslinien und Geschlechter im Vergleich. C: Körpergewicht beider Mauslinien und Geschlechter über die Zeit. D: Gewichtsverlauf des *M. quadriceps femoris* beider Mauslinien und Geschlechter über die Zeit (*: signifikanter Unterschied zwischen den Geschlechtern der Linie DU6P). E: Verlauf des Karkassengewichtes beider Mauslinien und Geschlechter über die Zeit.

Im Folgenden wurde die Aktivierung verschiedener Signalmoleküle im *M. quadriceps femoris* untersucht (Abbildung 2). AKT war in 29 Wochen alten weiblichen Mäusen der Linie DU6P auffällig stark an der Phosphorylierungsstelle Serin-473 aktiviert (232 % der Kontrolle, $p < 0,0001$). Wir untersuchten daher mögliche Mechanismen, die für die spezifische Aktivierung von AKT verantwortlich sein könnten. Der IGF-1-Rezeptor β als Teil des IGF-Systems zeigte keine Veränderung (132 % der Kontrolle, n.s.) und scheint keine entscheidende Rolle bei der AKT Aktivierung in unserem Modell zu spielen. Auch über Integrine bzw. über die Integrin-linked Kinase (ILK) konnten in Mäusen AKT-vermittelte Effekte auf Muskelansatz nachgewiesen werden (Boppart et al). Allerdings war die Expression von ILK im Muskel von weiblichen 29 Wochen alten Mäusen stark reduziert (18 % der Kontrolle, $p < 0,0001$) und kommt somit ebenfalls nicht als möglicher Faktor für erhöhte AKT Aktivierung in Frage. Myostatin, bekannt als negativer Regulator für

Muskelansatz, konnte signifikant erhöht nachgewiesen werden (125 % der Kontrolle, $p < 0,0001$) und kommt daher nicht als Mechanismus für Muskelansatz in 29 Wochen alten weiblichen DU6P Mäusen in Betracht. PTEN als negativer Regulator der AKT Aktivierung dephosphoryliert Phosphatidyl-inositol-3,4,5-triphosphat (PIP3) zu PIP2 (Das et al). Wir konnten in 29 Wochen alten weiblichen DU6P Mäusen in der Tat eine starke PTEN Abnahme nachweisen (58 % der Kontrolle, $p = 0,0061$), was zu einer AKT Aktivierung durch PIP3 Anreicherung führen kann. Zusätzlich fanden sich im Vergleich zwischen 11 und 29 Wochen alten DU6P Mäusen erhöhte Konzentrationen an phosphoryliertem PTEN (329 %, $p < 0,0001$), was einen Mechanismus zur Inaktivierung von PTEN darstellt. Es kann somit festgehalten werden, dass von allen untersuchten Mechanismen einzig die Kontrolle der Expression und Aktivität von PTEN als ursächlicher Faktor für die massive Erhöhung von AKT in weiblichen DU6P Mäusen in Frage kommen kann.

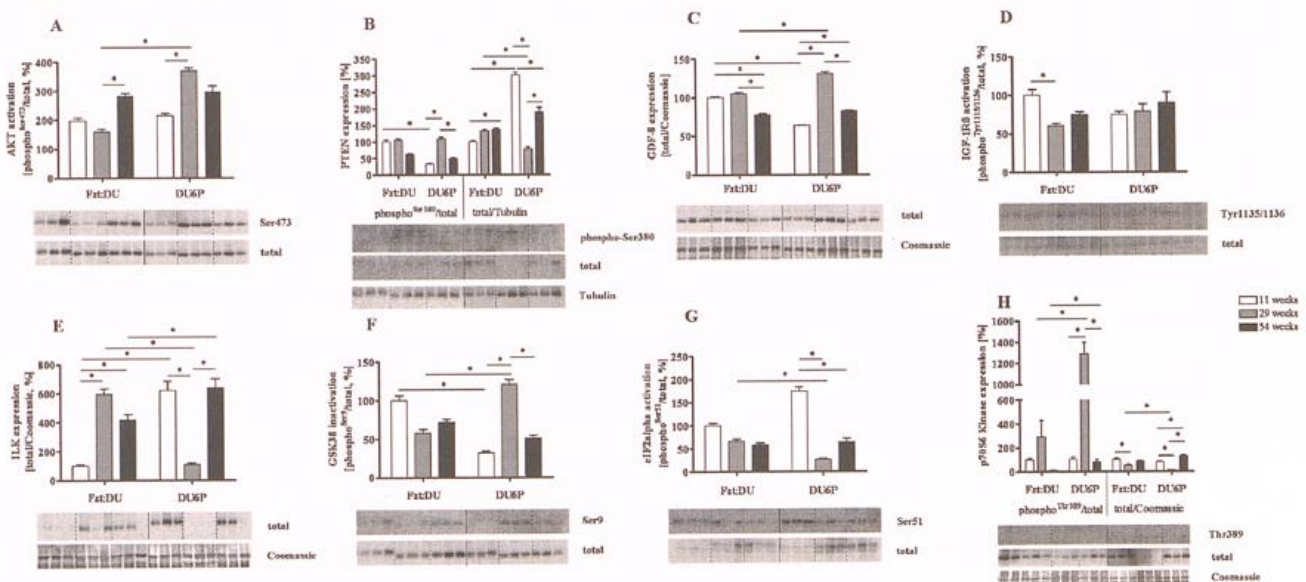


Abbildung 2: Analyse von Signalmolekülen im Muskel von weiblichen Mäusen der Linien Fzt:DU und DU6P im Alter von 11, 29 und 54 Wochen. Dargestellt ist das Verhältnis von Phosphoryliert/Total oder Total/Ladekontrolle (Ladekontrolle = Coomassie oder Tubulin) sowie die Western Blots.

Wir untersuchten weiter, welche Folgen die höhere AKT Aktivierung bei vermindertem PTEN haben kann und fanden signifikant erhöht phosphoryliertes GSK3β (208 % der Kontrolle, $p = 0,0469$). Diese Form ist inaktiv und führt zu einer Erhöhung der Glykogensynthese. Weiter konnte eine verringerte Aktivierung des eukaryotischen Initiationsfaktors 2α (eIF2α) gezeigt werden (41 % der Kontrolle, $p = 0,0210$), was zu einer Steigerung der Proteinsynthese führt. Ein Faktor, der zur Initiierung der Proteinsynthese an den Ribosomen nötig ist, ist die p70S6 Kinase. Diese scheint in weiblichen DU6P Mäusen

aufgrund einer signifikanten Verminderung eine untergeordnete Rolle zu spielen (20 % der Kontrolle, $p = 0,0422$). Zur Untersuchung des Glykogengehaltes im Muskel wurden sowohl chemische als auch histologische Analysen in weiblichen Mäusen im Alter von 29 Wochen durchgeführt (Abbildung 3). Der Glykogengehalt war in DU6P Mäusen ($0,12 \pm 0,02$ %) signifikant erhöht ($p = 0,0269$) im Vergleich zur Kontrolllinie ($0,04 \pm 0,01$ %).

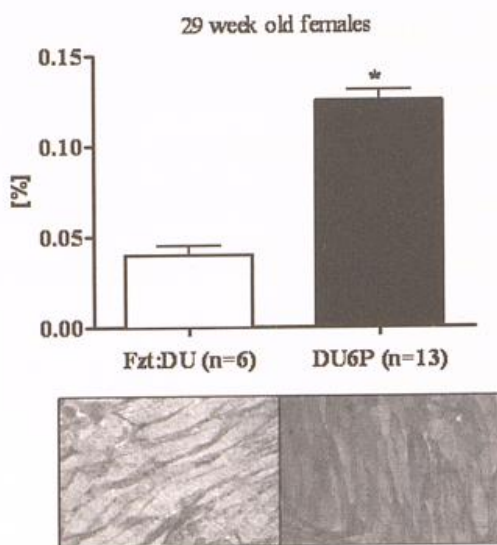


Abbildung 3: Muskel-Glykogen in 29 Wochen alten weiblichen DU6P Mäusen im Vergleich zur Linie Fzt:DU. Die Ergebnisse der histologischen Analyse bestätigen den Befund der chemischen Untersuchung.

Wir vermuten, dass die Aktivierung der Kinase AKT eine wichtige Rolle für den erhöhten Muskelansatz adulter weiblicher DU6P Mäusen spielt. Diese Aktivierung von AKT wird nach vorliegenden Ergebnissen möglicherweise durch eine Verminderung der Phosphatase PTEN verursacht. Bisher galt PTEN vor allem als Tumorsuppressorgen. Die Ergebnisse unserer Arbeit legen es nahe PTEN als neuen Biomarker für Muskelansatz zu diskutieren.

Die Kenntnis der physiologischen Regulationsprozesse von Muskelansatz in landwirtschaftlichen Nutztieren ist eine wichtige Voraussetzung für die Leistungsfähigkeit und Tiergesundheit und damit die Nachhaltigkeit tierzüchterischer Maßnahmen.

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Pooled samples for classification – What about PPLS-DA?

Anna Telaar¹, Kristian Hovde Liland², Dirk Reipsilber¹ and Gerd Nürnberg¹

¹Leibniz Institute for Farm Animal Biology, Dummerstorf, ²Norwegian University of Life Sciences, Ås

1 Introduction

Sample pooling is necessary for microarray experiments if not enough amount of RNA exists for the hybridization step or because of financial reasons. For classification studies like biomarker search, pooling should be avoided, because data is required at the "individual level" (Kerr 2003). Nevertheless pooled samples are used for biomarker discovery (Searfoss et al. 2003). Therefore Telaar et al. (2010) initiated a simulation study to analyse the dependence of the pooling effect on the structure of informative features, pool size and statistical learning method used. As measures of the pooling effect we use the prediction error and the number of important features simulated as informative for the classification. We investigate the statistical learning method power partial least squares discriminant analysis (PPLS-DA). PPLS-DA is a specialized version of the method partial least squares discriminant analysis (PLS-DA) which is a well known and powerful approach. We present a further optimization of the parameter choice of PPLS-DA for the single sample design and the pooling designs.

2 Materials and methods

Pooling: The gene expression of sample i for gene g , (with $g = 1, \dots, G$ and G depicting the number of genes) can be modelled on the log scale as $\log_2(u_{g,i}) = \mu_g + \varepsilon_{b_i} + \varepsilon_{t_i}$, $i = 1, \dots, n$ (with n number of samples) with mean gene expression level μ_g , biological and technical variation ε_{b_i} and ε_{t_i} which are independently, identically and normally distributed with mean zero and variances σ_b^2 and σ_t^2 . Pooling, the combination of several RNA samples, takes place before measuring the expression of the genes of these RNA samples. The gene expression of a pool p_j , $1 \leq j \leq n_p$ (with n_p number of pools), is the average of the samples which form the pool: $u_{g,p_j} = \log_2\left(\sum_{i=l+1}^{m+l} \frac{1}{m} 2^{(\mu_g + \varepsilon_{b_i})}\right) + \varepsilon_{t_j}$ where $1 \leq m \leq n$ is the number of mixture components within a pool, $l = s \cdot m$, $s \in \{0, \dots, n_p - 1\}$ is the starting index of a sample. The size of a pool is determined by m .

Design: Studying a classification task with two classes, our pools are built inside the classes.

Gene expression data simulation: Comparing a two-class classification study with a single sample design and pooling designs with pool size $m = 2, 3, 5$, we simulate 60 single samples per class and 1000 genes partitioned in an informative part and non-informative part for the classification. For the test set, 30 single samples per class are randomly chosen, which are used to calculate the prediction error (PE). The remaining 30 single samples per class are the basis for the different training sets.

The non-informative part of the data matrix which shows no differences between the two classes, consists of normally distributed random variables. Moreover, noise is added as technical variance in different proportions of the biological variance: $\{\frac{1}{8}\sigma_b^2, \frac{1}{4}\sigma_b^2, \frac{1}{2}\sigma_b^2, \frac{3}{4}\sigma_b^2\}$. We simulate 10 differentially expressed genes with a mean class difference randomly chosen between $[0.1, 0.5]$, these we name informative simulated features (ISF). We make 500 repetitions of the classification task.

PPLS-DA: For the classification we investigate the method PPLS-DA. We implement an inner 10-fold cross-validation to choose the optimal number of PPLS-DA components, with these components we apply a linear discriminant analysis. In contrast to the usual PLS-DA an optimization step is done during the calculation of the loading weights. For the gene expression data matrix $\mathbf{X} \in \mathbb{R}^{(30 \times G)}$ and the dummy matrix $\mathbf{D} \in \{0, 1\}^{(30 \times 2)}$ (indicating the class-membership of the classes $t = 1, 2$) the transformation matrix $\mathbf{t}_0(\gamma)$, $\gamma \in U$ can be factorized into: $\mathbf{t}_0(\gamma) = \mathbf{D}(\gamma) \mathbf{D}(\gamma)$.

Here $\mathbf{D}(\gamma)$ is a diagonal matrix with the standard deviation of the columns of $\mathbf{X}$ as non-zero entries exponentiated by $\frac{1-\gamma}{\gamma}$, $\mathbf{D}(\gamma)$ is a matrix which consists of the correlation of with exponentiated by $\frac{\gamma}{1-\gamma}$, and $\mathbf{D} = \text{diag}(\{\rho_t \mid t = 1, 2\})$, where ρ_t depends on the prior probability and relative group frequency of group t . Then the correlation between the transformed matrix $\mathbf{X} \mathbf{t}_0(\gamma)$ and $\mathbf{D}$ is maximized by using canonical correlation analysis. The power parameter γ is optimized normally over the interval $U = (0, 1)$, the endpoints 1 and 0 are checked explicitly. Considering the usual PLS-DA method, standard deviation and correlation part are coequal (same exponent 1) for the calculation of the loading weights. Further details of optimization and calculation of the loading weights vector are described in Liland et al. (2009).

Important features: As measure of importance for the classification we take the mean value of the loading weights over the components and order them according to their absolute values.

For the comparison of the single sample design with pooling designs, we consider the intersections of the set of ISF with the important features sets for the pooling designs using the set of important features for the single sample design as reference set.

3 Results

In Figure 1 the mean prediction error over 500 runs is shown for PPLS-DA for different proportions of technical variance and $\gamma \in [0,1]$ (dashed line) and $\gamma \in [0.9,1]$ (solid line).

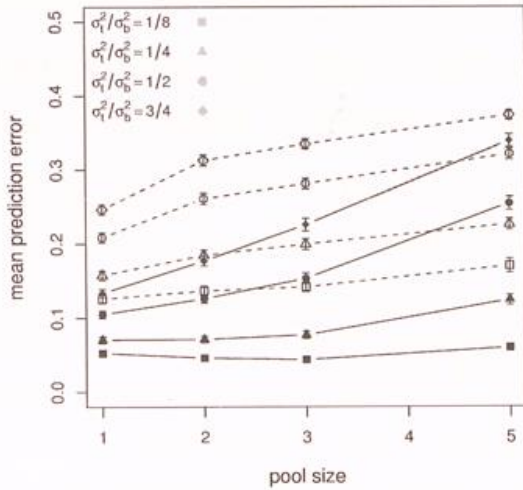


Figure 1: Mean PE with 95% confidence intervals for PPLS-DA, dashed lines and transparent symbols: $\gamma \in [0,1]$, solid line and filled symbols: $\gamma \in [0.9,1]$

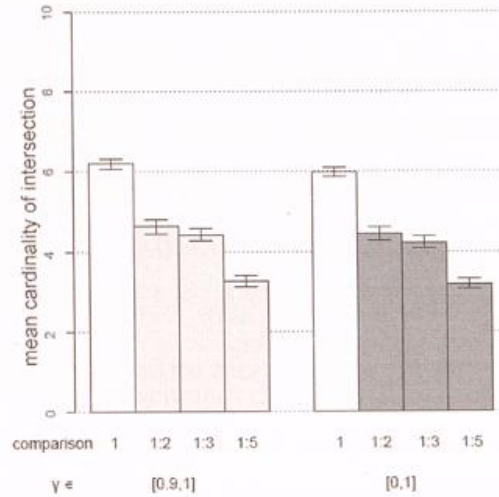


Figure 2: Cardinality of the intersection between important feature sets and the set of ISF

The results display a considerable lower prediction error (PE) for all designs for γ within the upper interval. Considering the results for $\sigma_t^2 = \frac{1}{4}\sigma_b^2$ as an example, the PE for $\gamma \in [0.9,1]$ ($\blacktriangle$) is nearly two times lower than for $\gamma \in [0,1]$ ($\triangle$) for the single sample design. Also for the pooling design, the PE is much lower for $\gamma \in [0.9,1]$. The PE is nearly equal, for designs with a pool size smaller than 3. Nevertheless for $\sigma_t^2 \geq \frac{1}{2}\sigma_b^2$, the PE increases stronger with increasing pool size for $\gamma \in [0.9,1]$.

Comparing the important feature sets for the classification, which is illustrated in Figure 2, no great differences are shown between the two power settings for γ for $\sigma_t^2 = \frac{1}{4}\sigma_b^2$. For the single sample design in average 6 of 10 ISF are important and of these sets between 4.5 and 3.2 are equal with the important features for the pooling design with $m = 2,3,5$.

The reason for the lower PE of PPLS-DA with $\gamma \in [0.9,1]$ is demonstrated in Figure 3 which

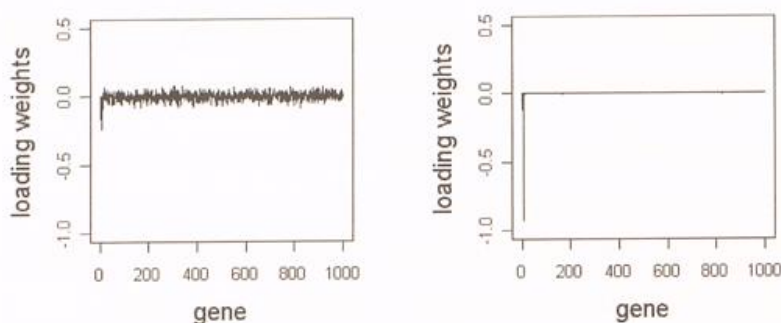


Figure 3: PPLS-DA loading weights for the first component (left) for $\gamma \in [0,1]$ and (right) for $\gamma \in [0.9,1]$ for the single sample design with $\sigma_t^2 = \frac{1}{4}\sigma_b^2$.

shows the loading weights for the first run for the single sample design with $\sigma_t^2 = \frac{1}{4}\sigma_b^2$ as an example. On the left side of Figure 3 the results are based on PPLS-DA $\gamma \in [0,1]$ where the optimization leads to $\gamma = 0.5$. Here also most of the non-informative features have loading weights equal to zero or near zero. On the right side of Figure 3 the result is shown for $\gamma \in [0.9,1]$ with optimal $\gamma = 0.9$. Almost all non-informative simulated features receive a loading weight near to zero, even smaller than for the left side, and differentially expressed genes with high mean class difference show large loading weights.

4 Discussion

The prediction error results for PPLS-DA are considerably improved by constraining the optimization interval of γ to the highest values ($[0.9,1]$) for the single sample design and all pooling designs. Therewith PPLS-DA, $\gamma \in [0.9,1]$ clearly outperforms all other methods used in Telaar et al. (2011) for the classification with differentially expressed genes. Using this setting, the non-informative genes, which could be considered as noise, get a loading weight near to zero and have therewith nearly no influence on the calculation of the PPLS-DA components which are used for a linear discriminant analysis. On the contrary, the values of the genes which have a high correlation to the vector of the group memberships receive larger loading weights.

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Including metabolomic profiles to improve genetic value prediction: an integrated bioinformatics approach using weighted genome-wide marker information

Nina Melzer, Dörte Wittenburg and Dirk Repsilber

¹Leibniz Institute for Farm Animal Biology, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

1 Introduction

We develop an integrative bioinformatics approach to improve the prediction of milk phenotypes from genome-wide marker data using metabolite profiles together with a weighting heuristics, based on a realistic simulation study. Towards this objective, we adopt an approach from the field of genomic selection (e.g. Meuwissen [1]). In this field, simulated genome-wide marker data and phenotypes are used to develop methods for predicting genetic values, but to date there does not exist a strategy to include additional information from functional levels of gene expression, such as for example metabolite levels. In our study, we integrate the metabolome level and employ a weighting for the SNP data. The metabolome level is simulated using the metabolism SBML model 70 from Holzhütter [2]. Metabolite profiles can be used to predict a milk phenotype trait using methods of the field of machine learning. Here, several methods can be used together with OMICs profiles, for example Random Forest [3]. From the trained learning machine it is possible to extract the important variables for phenotype prediction. These measures of importance can be used after locating the belonging enzymes, which catalyze the metabolite reactions of the important metabolites. From the identified enzymes it is possible to go further back to the genome level and weight SNPs in the corresponding gene region of the enzyme. The weights and the genome-wide marker data are jointly used to predict the genetic value. For our approach, we applied the extended fastBayesB method [4, 5]. In this study, it is exemplarily tested if and to which degree the weighting approach leads to an improvement of the genetic value prediction.

2 Materials and methods

Data sets: We use ten simulated data sets as described in Melzer et al. [6]. We build our analysis on the genome-wide marker data and on metabolite profiles. We simulate the phenotypes depending on three different values of broad-sense heritability. Further, the training generation and test generation have a

population size of 2000 cows. For this study, we use 52273 simulated SNPs, thereof 230 QTL are used to simulate a genetic value by determining kinetics of 230 enzymes. These enzymes catalyze 270 metabolites, which are simulated using an SBML model as in Melzer et al [6]. The equilibrium levels of these metabolites are then used to simulate a genetic value. No additional noise is added to the metabolite profiles.

Workflow: To receive a weighting scheme for predicting the genetic values, several steps are necessary as shown in figure 1. The first step is to use the metabolite profiles to predict the phenotype using the Random Forests algorithm which employs techniques of variable selection. We apply Random Jungle from Schwarz et al. [7] (version 0.7.2, default settings). The variable importances for each metabolite quantify how much of the information of the metabolite profiles can be used to explain the phenotypes. The variable importances are used for the second step: We know which enzymes catalyze the reactions involving the important metabolites. Here, it is possible that one or two metabolites are catalyzed from the same enzyme. If two metabolites are catalyzed, the maximum of the importance values is used as weight. For the simulated SNPs we use the „real“ positions on the genome (Illumina 50k SNP chip) and create a vector of weights for 52273 SNPs where the importance values are assigned to the QTL positions. All other SNPs are weighted with the neutral value „1“. In the third step, we estimate genetic effects for each SNP with the fastBayesB method including additive and dominance effects [4]. The vector of weights is incorporated in the style of a weighted regression approach. Eventually, the genetic value is predicted as the sum of genotypic effects for each animal.

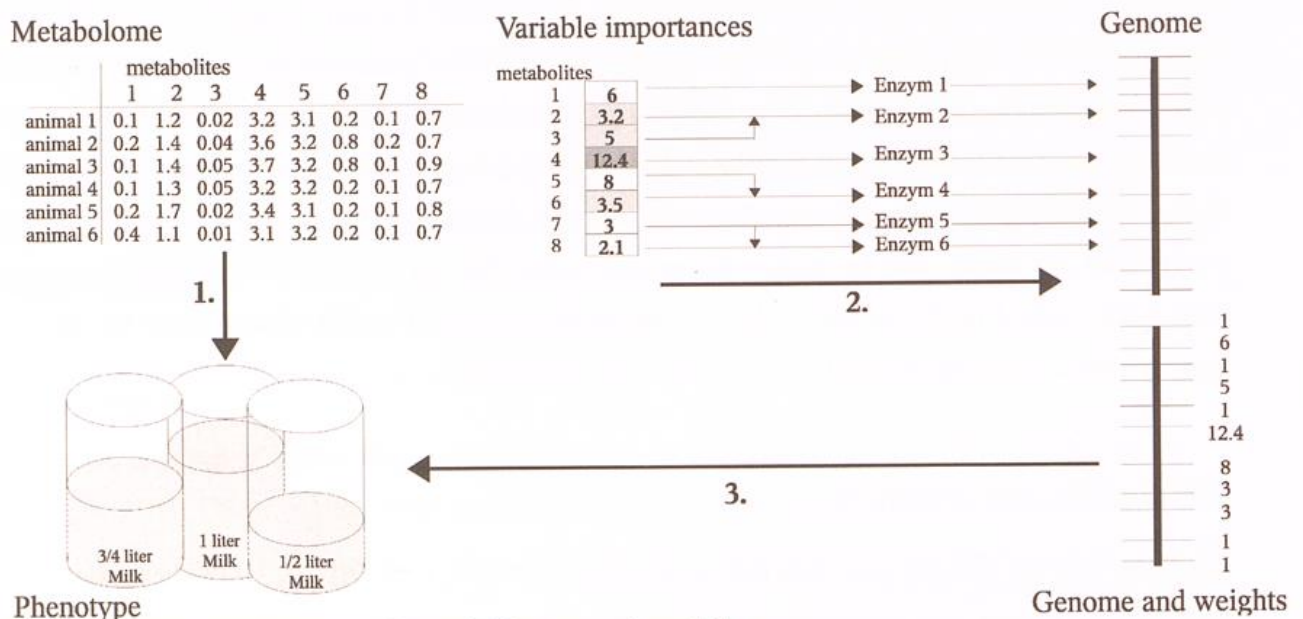


Figure 1: Schematic representation of the weighting approach workflow.

Weighting scenarios:

scenario 1: All 230 QTL positions were weighted with a constant weight and all other SNPs were weighted with “1”. Weights were elements of $\{1, 2, \dots, 10\}$. As a result we measured the correlation between true (simulated) genetic value and its prediction, which is called precision. Figure 2 shows that a weight of 2 is an appropriate value to improve the prediction of the genetic value.

Scenario 2: The 270 simulated metabolites were handled as measured and identified metabolites, and all were used for steps 1 and 2 of the weighting approach. The weights (variable importances) for 230 QTL were obtained from Random Forest.

Scenario 3: Only 20% of the 270 metabolites were selected randomly as “identifiable”, requiring that metabolite 13, which was important for the simulated phenotypes, was always included. 80% of the metabolites were considered “non-identifiable” and were simulated at random.

Scenario 4: Analogously to scenario 3, but completely without metabolite 13.

For scenario 2-4: The observed importance values (iv) were scaled as follows: $iv_{scale} = \exp(iv/\max(iv))$, where exp is the exponential function. After this transformation, the weights lie in a range from 1 to 2.718, which was shown to be appropriate in scenario 1. To compute a vector of weights two strategies were implemented: (1) all “identifiable” metabolites were used; or (2) the top 10% “identifiable” metabolites only.

3 Results

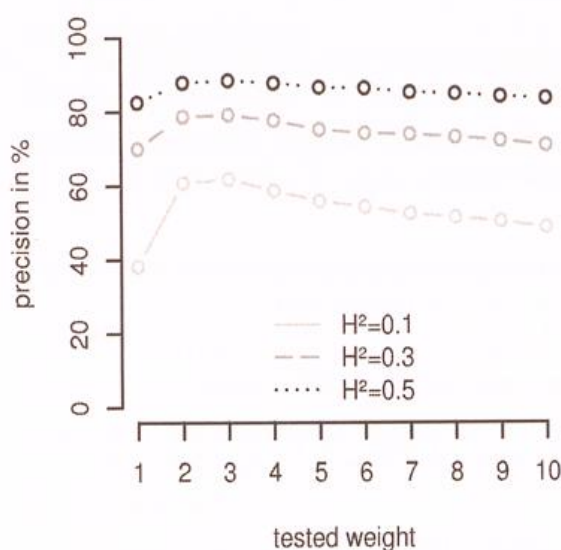


Figure 2: precision of genetic value prediction for different values of weights

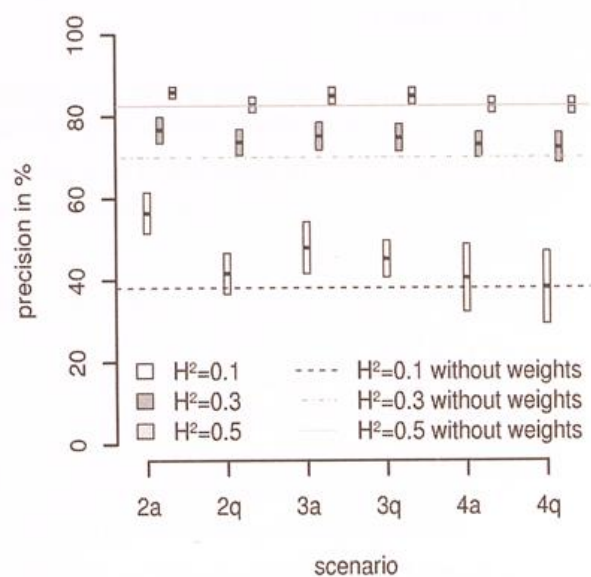


Figure 3: predicting precisions (mean $\pm$ standard error) for scenarios 2-4 depending on proportion of “identifiable” metabolites: “a” means all “identifiable metabolites, “q” the best 10% “identifiable” metabolites.

Figure 3 shows the predicting precisions achieved for scenarios 2-4 and for the two selection strategies. It is obvious, that in scenario 2, where all “identifiable” metabolites (a) were used, we achieved the best predicting precisions for all used broad-sense heritabilities and tested scenarios. Further, when the best “identifiable” metabolites (q) were selected for the different scenarios, the predicting precisions were decreased in comparison to the case where all “identifiable” metabolites were used. Additionally, in comparison to precisions achieved without using the weights (see figure 3), in most scenarios with weighting a higher predicting precision was achieved.

4 Discussion

Our simulation results demonstrate the feasibility of an integrative bioinformatics approach to enhance phenotype prediction based on genome-wide marker data by incorporating information about the metabolome level, based on a weighting approach as a matter of principle. Choice of simulation parameters is on the one hand designed to meet experimental data structures (sample size, SNP distribution, number of QTL and metabolites). However, on the other hand, many simulated details of the genotype-phenotype map remain artificial choices, and, hence, a proof of principle using experimental data is necessary and planned for our existing experimental data. The results of our simulation study are encouraging, and we envision testing further possibilities of optimisation, e.g. regarding the actual re-scaling of the variable importances to yield the weighting vector, using experimental data.

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Bayes-method for mapping imprinted QTL in an inbred F₂-design

Ulrike Borchardt¹ and Norbert Reinsch¹

¹Leibniz Institut for Farm Animal Biology, Dummerstorf

1 Introduction

Most QTL studies in pigs involve additive and dominant effects but imprinting is often ignored. Genomic imprinting refers to a phenomenon in which the same genes are expressed differently, depending on their parental origin. It has been shown; that between 5% and 19% for traits connected to growth and body components of the total additive genetic variance was controlled by imprinted loci [Neugebauer]. To find additive, dominance and imprinted QTL we used a multiple QTL model estimating all effects simultaneously in a single model. We implemented an efficient and adapted Bayesian method with genomic imprinting to estimate all effects (based on [Xu]). This method is also an essentially model-selection-free method. Small effects get a small weight so that their inclusion should have negligible effects on the final results.

2 Materials and methods

Statistical methods:

For an F₂ population the mixed model is:

$$y_i = \mu + V_i d + \sum_{j=1}^p (X_{ij}a_j + W_{ij}b_j + Z_{ij}c_j) + e_i,$$

Let y_i for $i = 1, \dots, n$ be the phenotypic value of the i th animal, μ is the population mean, p is the number of possible QTL. V_i is the design matrix of the fixed effects d . X_{ij} , W_{ij} and Z_{ij} are dummy variables, which coded the genotype of the j th QTL and i th individual. The QTL effects of QTL j are a_j (additive), b_j (dominant) and c_j (imprinting). E_j is the residual error, distributed as $N(0, \sigma^2)$.

To estimate the additive, dominant and imprinting effects, we applied the model-selection-free method of (Xu). The multiple marker analysis and the linear model were extended by imprinting and fixed effects. Xu's method uses that each marker effect gets a normal prior

with mean zero and a unique variance. The effect-specific prior variance was estimated from the data.

X_{ij} , W_{ij} and Z_{ij} are defined for genotype A_1A_1 : $\sqrt{2}$, -1 and 0 . For a genotype A_1A_2 : 0 , 1 , $\sqrt{2}$, for A_2A_1 : 0 , 1 , $-\sqrt{2}$ and for A_2A_2 : $-\sqrt{2}$, -1 and 0 . The coded variables are independent and have a zero expectation and a unity variance. The regression coefficients are defined as $a_j = G_{11} - G_{22}$ for additive effects, $b_j = G_{12} + G_{21} - G_{11} - G_{22}$ for dominant effects and $c_j = G_{12} - G_{21}$ for imprinting effects, where G_{11} , G_{12} , G_{21} and G_{22} are the four genotypic values.

The likelihood function is the distribution of the observables (phenotypes and marker information) and the function of the unobservables (effects and variances). The prior distribution is the distribution of the unobservables. In the MCMC-implemented Bayesian analysis, we sample the unobservables from the above joint posterior distribution.

Animals and analyzed traits

In this study, we used a total of 645 F_2 pigs, derived from Duroc and Pietrain founder animals. All animals were kept at the Frankenhorst experimental research farm at the university of Bonn (Germany). The phenotypes of all F_2 pigs were recorded in an abattoir, according the guidelines of German performance stations. In total 26 traits were analyzed. On the one hand, the traits describe carcass compositions and on the other hand the meat quality.

125 microsatellites and 6 SNP markers were used to genotype the animals and covered 89% of these. The CRI-MAP 2.4 software recalculates the gender-average linkage map. Gencheck and Pedcheck (version 1.1) checked genotypic errors. More detailed information can be found in (Liu) and (Grosse-Brinkhaus).

3 Results

In this study, we have 131 markers on the 18 autosomes. We calculated genotype probabilities for 244 possible QTL positions. A large part of these are uncertain genotypes. For the mixed model we use different fixed effects like birth or slaughter months, family, gender, carcass weight and age. As default initial values all QTL effects were set equal zero and all variances equal to 0.5. The length of Markov chain is 110000 sweeps. 10000 sweeps were deleted (burn-in period). The observations were added and the posterior mean and variances of QTL effects are calculated.

In the Bayesian analysis markers with small effects are shrunk towards zero. We performed several analyses to check the results. We see that the peaks swing around special points. To have significance thresholds to distinguish QTL from non-QTL, we randomly shuffled the phenotypes (permutation test) [Sillanpää].

Table 1: Significant effects

Chromosome	Markers	Phenotype	Additive effect	Dominance effect	Imprinting effect
SSC 1	SW1851	Ph _{24h} ham	-0.021		
	SW2166	dressing			0.207
	S0113 – SWR2182	Ph ₄₅ loin		-0.005	
		Ph decline		-0.019	
		dressing			-0.260
		Ph _{24h} ham			0.014
SSC 3	S0164	Thawing loss (g)		0.0513	
SSC 5	SW1134 – S0005	Conductivity 24h ham			1.970
	S0005	Conductivity 24h ham			-1.940
SSC 6	S0087 – SW1067	Estimated belly lean content (%)	0.148		
		Ratio of fat to muscle area	-0.002		
		Loin eye area above M. long. dorsi (cm ²)	1.056		
		Bonner formula (%)	0.547		
	S0300 - CKM	Estimated belly lean content (%)			-0.147
		Loin eye area above M. long. dorsi (cm ²)			-0.078
		Bonner formula (%)			-0.478
		Backfat loin (cm)			0.060
	S0220 – S0059	Backfat shoulder (cm)	-0.081		
	S0059 – S0003	Cooking loss	0.509		
SSC 7	S0102 – SW175	Carcass length (cm)	-0.028		
SSC 13	SW344	Conductivity 45 min loin		-0.012	
SSC 14	SWC27	Drip loss		0.437	-0.659
SSC 17	SW840 – SW2431	Side fat thickness (cm)	0.0288		
		Ratio of fat to muscle area	0.0009		
		Backfat loin (cm)		-0.143	
		Estimated belly lean content (%)	-0.414		

Table 1 is sorted by chromosome and position within each chromosome. Additionally, the significant additive, dominance, and imprinting effects of each QTL are listed. A total of 26 QTL for 17 of the 26 traits were found to be significant at 5% genome-wise levels.

Additive effects are often on chromosome 6 and 17. Dominant effects have a cluster on chromosome 5 and 16. But many possible QTL effects are under the threshold (especially carcass composition traits). Similar to the dominant effects the imprinting effects on chromosome 2 are under the significant threshold. Especially the fat components between markers SW1879 and SW1844 are present. The second cluster on imprinting effects is on chromosome 6. The phenotypes dressing and Conductivity 24h ham have opposite direction effect in a small interval and either indicate two QTL with imprinting effects in repulsion are an artefact due to large monte carlo errors.

4 Discussion

Simultaneous QTL-mapping is very time expansive, because imprinting genotypes are uncertain. In every iteration many uncertain genotypes have to be imputed. The comparison of results with other studies is not easy, because the special F₂ population of Duroc and Pietrain and the genome-wise level (most paper have chromosome-wise level and detected more QTL). In direct comparison with the same population and single-QTL-models we detected the same QTL on chromosome 1 ph 24h ham and loin, on chromosome 6 the estimated belly lean content and on chromosome 7 the carcass length [Liu]. We see that our analysis yields similar QTL position in comparison with other studies (additive and dominance effects). Therefore we can accept the imprinting effects (positions), especially the cluster on chromosome 2 (not significant) and 6. Genomic imprinting can be interpreted as an interaction effect between alleles and parental origin with locus. Identification of interactions between different loci – additive-additive, additive-dominant, dominant-additive, dominant-dominant – is even more demanding from a computational point of view.

5 Literature

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Vergleich der Güte verschiedener Pooling-Designs bei Tests auf differentiell exprimierte Gene

Henrik Rudolf, Norbert Reinsch

Leibniz-Institut für Nutztierbiologie Dummerstorf, Forschungsbereich Genetik und Biometrie

1 Einleitung

In Genexpressionsstudien ist Pooling, das Mischen der mRNA mehrerer Individuen, eine weit verbreitete Methode. Wissenschaftler greifen auf dieses Mittel zurück, um unzureichende RNA-Mengen vor der Hybridisierung auszugleichen, oder die Kosten des Versuchs durch eine Verringerung der Anzahl der Arrays zu senken. Peng *et al.* (2003) schlussfolgern, dass optimale, gepoolte Designs wählbar sind, so dass die Einhaltung statistischer Voraussetzungen einhergeht mit einer Reduktion der Kosten der Versuche. Zhang *et al.* (2007) gelang es, die theoretisch positive Differenz zwischen den zu erwartenden Expressionswerten eines Pools und einem Mittel der beteiligten Individuen (Jensensche Ungleichung) auf der Log-Skala zu approximieren, wie auch die Varianz von Pools gleicher Größe. Bisher wurde empfohlen, während eines Versuches die Poolgröße nach Möglichkeit nicht zu verändern. Erstens, weil dies zu Verzerrungen in den Parametern führt und außerdem die Varianz-Homogenität in den Daten verletzt.

Bei Versuchen in denen nach Tieren mit seltenen Eigenschaften gesucht wird, ist jedoch vor Beginn nicht klar wie viele Subjekte zu dieser Gruppe gehören. Daher ist bei fest vorgegebener Anzahl von Arrays flexibles Pooling mit Größen entsprechend der gefundenen Individuen je Versuchsdurchgang wünschenswert. Wir analysieren diese Pooling Experimente und behandeln die bereits erwähnten Nachteile. Dazu wird im Design jedem Pool einer Behandlung ein gleichgroßer Pool von Kontrollen gegenüber gestellt. Es lässt sich zeigen, dass dann die Bedingung für unverfälschte Kontraste bei bestimmten Strukturen der Kovarianzmatrix erfüllt ist. Zur Untersuchung der neuen Methode in der Analyse haben wir Experimente, die die beschriebenen Voraussetzungen an Daten und Kovarianz umsetzen, simuliert. Im Folgenden werden erste Ergebnisse präsentiert, welche anhand der Güte der Tests Rückschlüsse auf Einsparmöglichkeiten bei der Zahl der Arrays gestatten.

2 Methoden

Wir erweitern das lineare Modell der Log-Intensitäten durch die Verzerrungen $h \in \mathbb{R}^n$, $y = X\beta + h + e$ (Glg. 1), und sei zunächst $V = I\sigma_e^2$. Hierbei enthalte der Vektor h die durch das Poolen verursachten Verzerrungen h_i , wobei Beobachtungspaare mit gleicher Poolgröße (γ_i) jeweils die gleiche Verzerrung aufweisen.

Dann lässt sich zeigen, dass die Verzerrungen auf der Ebene der Parameter ausgeglichen werden, falls für jede auftretende Poolgröße ein gleichgroßer Kontrollpool gewählt wird. Eine kleinste Quadrate Schätzung für die festen Effekte im linearen Modell erhält man durch: $\hat{\beta} = (X'X)^{-1} X'y$. Der Erwartungswert von $\hat{\beta}$ unter Berücksichtigung von h in Gleichung (1) ist dann $E(\hat{\beta}) = (X'X)^{-1} (X'X)\beta + (X'X)^{-1} X'h$. Hier wird die Designmatrix benutzt, um den Parametern die in den einzelnen Beobachtungen enthaltenen Verzerrungen $\tilde{h} := (X'X)^{-1} X'h$ zu zuordnen. Sei der Kontrast $K'\tilde{\beta}$ eine schätzbare Funktion. Diese hat dann den Erwartungswert $K'\beta + K'\tilde{h}$ und ist unverzerrt schätzbar, falls $K'\tilde{h} = 0$ ist.

Aufgrund der Varianzheterogenität brauchen wir für die Simulation und Auswertung dieser Versuchspläne eine verallgemeinerte Darstellung von V in (1).

Zunächst simulieren wir individuelle Log-Intensitäten mit normal verteilten Fehlern mit Varianz σ_b^2 . Nach Transformation werden diese Werte gemittelt mit zufälligen Gewichten (mit Variabilität σ_z^2) und dann wieder auf der Log-Skala der technische Fehler (σ_t^2) addiert.

Mit der Theorie der verallgemeinerten linearen Modelle (V beliebig) werten wir die simulierten Log-Intensitäten aus. Dazu nutzen wir die Approximation von Zhang et. al. für die Varianz von Pools auf der Log-Skala $v_{ii} = \sigma_t^2 + \left(e^{\sigma_b^2} - 1\right) \frac{1}{\gamma_i} + \left(e^{\sigma_b^2} - 1\right) \sigma_z^2 \frac{\gamma_i - 1}{\gamma_i^2}$. Die empirische Güte ermittelt sich durch die Anzahl der abgelehnten Hypothesen $K'\beta = \Delta = 0$ auf der Alternative, wobei verschiedene mittlere Klassenunterschiede Δ gewählt wurden. Mit steigender Zahl von Pools (ebenfalls mit vorgegebener zufälliger Größe) lässt sich dann die Eignung der untersuchten Designvarianten abschätzen. Vorwiegend ist diese ersichtlich, an welcher Stelle die Wahrscheinlichkeit für den Fehler 1. Art ($\alpha = 0.05$) bei der Erkennung differentiell exprimierter Gene unterschritten wird. Dazu wurde das beschriebene Szenario für Designs mit Einzelproben, mit Pools gleicher Größe für den gesamten Versuch und mit flexiblen Poolgrößen betrachtet.

3 Ergebnisse

Anhand der F-Tests für fixe Effekte werden die statistischen Eigenschaften der Designs (ermittelt mit 10000 Wiederholungen je Poolgröße) dargestellt. Die Güte als Rate der Ablehnungen der Hypothese “Es liegen keine Unterschiede zwischen den Gruppen vor“ auf der Alternative ist in Abb. 1 abgetragen zur Anzahl der Pools von vier bis 50.

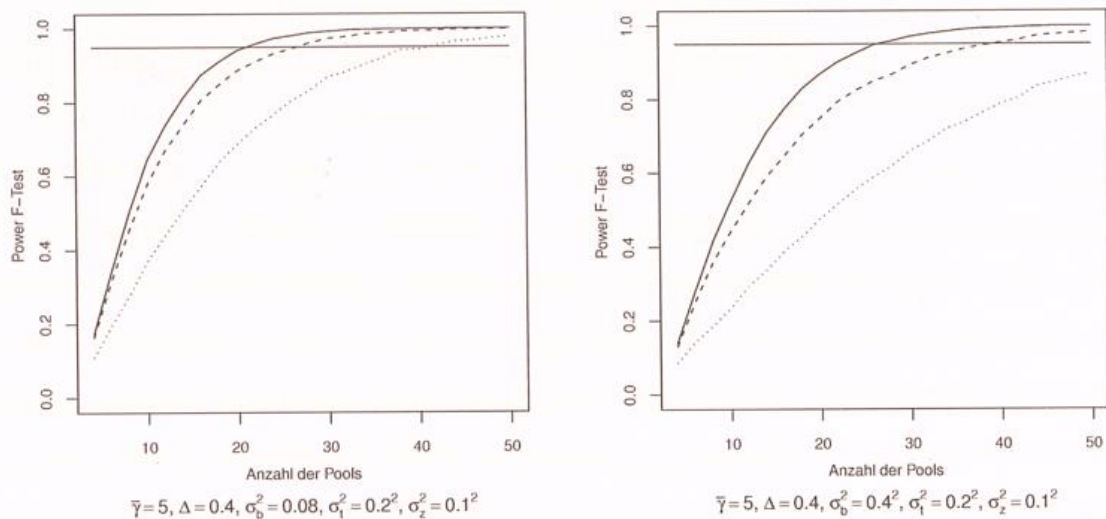


Abb.1: Vergleich der empirischen Güte der Designs mit Einzelproben (gepunktete Linie), mit gleichen (gestrichelt) und flexiblen (durchgezogen) Poolgrößen. Varianzverhältnisse biologisch zu technisch sind zwei (links) bzw. vier (rechts).

Die Kurve für das flexible Pooling-Design steigt schneller an und erreicht früher das 95%-Niveau als im Fall gleicher Poolgrößen. Außerdem untersuchen wir den Einfluss der Pool Variabilität, der Abweichungen vom idealen Pool (d.h. identische Gewichte).

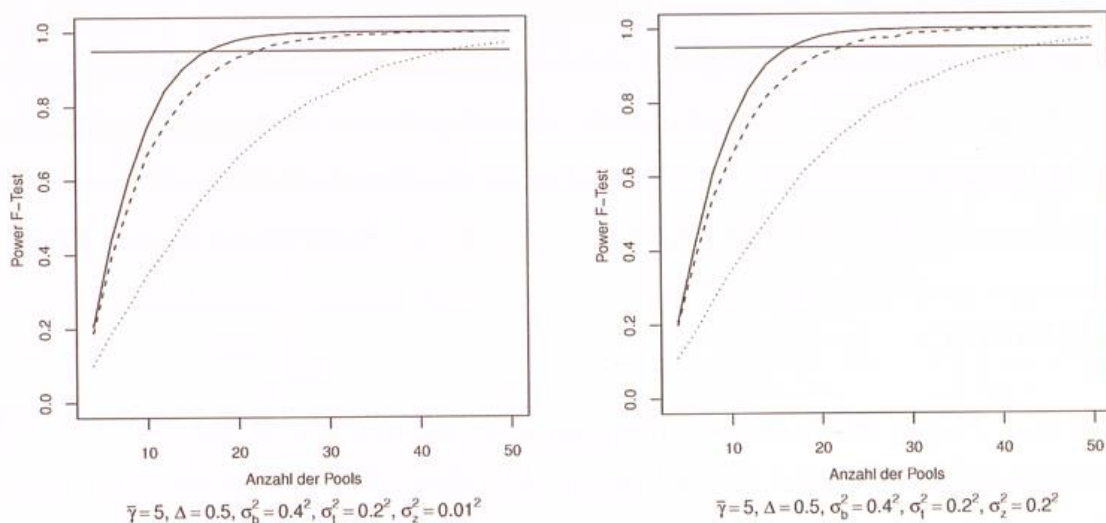


Abb.2: Vergleich der Güte der drei Designs bei Veränderung der Pool-Variabilität (Legende wie Abb. 1)

Bei Erhöhung dieser findet sich ein leichter Rückgang in der Präzision, der zu einer verringerten Güte führt. Dieser Unterschied ist sehr klein, falls die biologische Varianz aus

dem niedrigen Bereich der Parameter gewählt wird. Bei erhöhter biologischer Variation zeigt sich in Abbildung 2 ein erkennbarer Effekt.

4 Diskussion

Die vorliegende Simulationsstudie zeigt, dass die Güte bei gleicher Zahl von Pools (bzw. Arrays) für die Designs mit flexiblen Poolgrößen höher als im Fall gleichgroßer Pools ist. Da man für jenes Design nur das Minimum der auftretenden simulierten Poolgrößen nehmen kann, können nicht alle Individuen einbezogen werden. Besonders effektiv ist die Anwendung der neuen Methode, wenn die Kosten der Versuchsobjekte im Verhältnis zu den Kosten der Arrays klein sind. So wird in Abb. 1 links die 95%-Schwelle bei flexiblen Poolgrößen bei 22 hingegen bei gleichgroßen Pools erst bei 28 Pools erreicht. Dieser Effekt wird verstärkt bei größerer biologischer Varianz (Abb. 1). Hier kann theoretisch mit einer Einsparung von bis zu 20% der Pools (Arrays) bei gleicher Güte und identischen Hybridisierungskosten gerechnet werden. Die Einzelproben Designs werden von beiden Pooling-Methoden klar übertroffen.

Der Effekt der technischen Poolvariabilität wurde von Zhang *et.al.* (2007) aufgrund eines Fehlers in der Approximation unterschätzt. Nach Korrektur bleibt der Einfluss gering, lediglich in Experimenten mit höherer biologischer Varianz zeigt sich eine leicht verringerte Güte mit steigender Variabilität.

Das Prinzip der Konstruktion von Versuchsplänen mit flexiblen Poolgrößen lautet: ausbalancieren der Poolgrößen für jeden Behandlungsvergleich durch gleichgroße Kontrollpools. Dadurch kann zwischen Blöcken von Versuchen die Poolgröße der Behandlung frei gewählt werden, wenn entsprechende Anzahlen von Kontrollen in den gegenüber zu stellenden Pool eingehen. Diese Bedingung ist wesentlich flexibler als die bisherige Vorgehensweise. Die Umsetzung der Versuchspläne ist bei Experimenten mit großer Anzahl von Kontrolltieren leicht möglich. Im Fugato-plus Projekt HyBee ist dies z.B. der Fall bei der Untersuchung des Hygieneverhaltens von Bienen innerhalb eines Volkes.

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Genome-wide association and expression analyses reveal candidate genes for leg weakness in pigs

Noppawan Rangkasenee¹, Eduard Murani¹, Ronald Brunner¹, Karl Schellander², Henning Luther³, Andreas Hofer³, Siriluck Ponsuksili¹, and Klaus Wimmers¹

¹Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany, ²Institute of Animal Science, University of Bonn, Bonn, Germany, ³SUISAG, Sempach, Switzerland

1 Introduction

Leg weakness in pigs is one of the most serious problems in pig production increasingly causing culling of breeding pigs, which results in economic losses and affects animal welfare (Fukawa and Kusuhara, 2001, Yazdi et al., 2000). Leg weakness is any lesion or dysfunction in the leg or joint that may cause clinical signs of lameness. Pigs have a high growth capacity of skeletal muscles and hence are per-se pre-disposed to suffer from pathologic disorders of the osteochondric system because of a mismatch between the developmental maturation of the skeletal system and the direction and magnitude of muscle force on the cartilage, the epiphyseal plate and the sub-chondric bones (Bickhardt, 1998; Uhlhorn et al., 1995). Accordingly, leg weakness in pigs can be mostly attributed to osteochondrosis, OC. These pathological aberrations are the result of disturbances in the differentiation of the chondrocytes. The heritability of leg structure traits was low to moderate ranging from 0.10–0.50 (Reiland et al., 1978; Lundeheim, 1987; Stern et al., 1995; Jørgensen and Andersen, 2000). There are positive phenotypic and genetic correlations between linear scores of visually appraised leg conformation and joint lesions scores (Fukawa and Kusuhara, 2001). Extreme leg conformations are predisposing factors for the development of OC. Several candidate genes have been proposed to be causative for OC in different breed of pigs (Onteru et al., 2008; Fan et al., 2009). In this study, we aimed to identify candidate genes relevant to predisposition for leg weakness by using a genome-wide scan and microarray expression.

2 Materials and methods

Genome-wide association analysis (GWA): A total of 298 commercial pigs phenotyped by SUISAG for conformation of feet and leg structure traits and OC lesions were used in this study. After slaughter, elbow and knee joint were collected for examination. All joints were observed in specified locations; head of humerus (HH), condylus medialis humeri (CMH),

condylus lateralis humeri (CLM), radius and ulna proximal (RUP), distal epiphyseal cartilage of ulna (DEU), head of femur (HF) and condylus medialis femoris (CMF). OC lesions were scored in a range from 1 (normal joint) to 4 (severe joint lesions), with the exception of DEU and CMF which were scored from 1 (normal) to 6 (severe lesions). Overall lesions scores were summed up (OC_sum) and animals were assigned to two categories: high and low lesion scores (OC_cat corresponding to $OC_sum \geq 12$ vs. ≤ 8). The PorcineSNP60 BeadChip (Illumina, Inc.) was used for genotyping. The association analysis between SNPs and OC scores at each joint (OC_HH etc.), the sum of scores of all joints (OC_sum), and OC category (OC_cat) was done by ANOVA (JMP Genomics, SAS institute), with line, sex and genotype as fixed effects, and body weight as covariate.

Gene expression analysis: Discordant sibpairs for OC lesions of a F2 resource population based on Duroc and Pietrain (DuPi) were used. The OC lesions were histopathologically monitored and classified into five cartilage condition scores according to the severity of OC lesions at the Institute of Animal Science, Bonn. For expression analysis, 22 samples of articular cartilage tissue from CMH and CMF were used. Total RNA was isolated by TRI Reagent (Sigma, Taufkirchen, Germany) according to the manufacture's protocol. The expression patterns were produced using Affymetrix GeneChip Porcine Genome Array (Affymetrix). The quality of hybridization was assessed and data were processed with MAS5.0 (Expression Console, Affymetrix) to generate cell intensity files (using default settings with detection P-values of <0.04 for 'present', ≥ 0.04 and ≤ 0.06 for 'marginal', <0.06 for 'absent'; only 'present' calls were used). Quantitative expression levels of the present transcripts were estimated using PLIER (Probe Logarithmic Intensity Error, Expression Console, Affymetrix) for normalization. The statistical analysis was done by JMP Genomics Software (SAS institute). Analysis of variance was performed to detect genes differentially regulated at $P < 0.05$. The Ingenuity Pathways Analysis (IPA) (Ingenuity System) applications were applied with current annotation of 20,439 probe set (EnSEMBL Sscrofa 9) (Naraballoh et al., 2010), and were used to assess relevant biofunctions.

3 Results and discussion

In CMH and CMF tissues 2,219 transcripts were differentially expressed depending on OC score ($P \leq 0.05$). Among these, the top ranked biological functional of genes included cellular growth and proliferation, skeleton and muscular disorder, genetic disorder, vitamin and mineral metabolism, cell death, connective tissue development and function, tissue development, cell cycle, and protein degradation (Fig 1). These functions are related to the

ossification of bone, development of growth plate, differentiation of chondrocytes and osteoclasts, and cell apoptosis that play an important role in the development of OC (Ytrehus et al., 2007).

The GWA analysis using the threshold of $q \leq 0.05$ showed 7 SNPs that were associated with joint lesions. For OC_cat there were 4 SNPs which located on SSC5, 10, and 18 (Table 1). Three SNPs on SSC14 showed the association with OC lesion at CMH and CMF. In addition, at $q \leq 0.1$ displayed regions on SSC5, 10, 14 and 18. In the candidate region on SSC5, *TMCC3* (transmembrane and coiled-coil domain family 3) located 0.11 Mb upstream of SNP (*MARC0069757*). Two SNPs on SSC14 (*ASGA0062794* and *MARC0002724*) mapped close to *TBX5* (T-box 5) and *MED13L* (mediator complex subunit 13-like), respectively. A SNP on SSC10 (*ALGA0057003*) was upstream of *MARK1* (MAP/microtubule affinity-regulating kinase 1). These genes are represented among the differentially expressed genes related to OC. Functional of these genes are classified into cell proliferation, differentiation, and organisation that are related to OC development. The integration of gene expression and GWA analysis provides a list of functional positional candidate genes for the predisposition to develop leg weakness in pigs.

Table 1 Genomic regions associated with OC lesions in commercial pig herds.

SNP	Gene Location (Mb, SSC)	Gene within 1 Mb	Gene Location (Mb, SSC)	q-Value	Traits Association
M1GA0019792	144.9 (14)	PTPRE	144.3 (14)	0.0038 , 0.0904	OC_CMH, OC_sum
MARC0007896	15.7 (18)	LOC414428	15.6 (18)	0.0577	OC_CMH
MARC0024273	7.4 (18)	BRAF	7.4 (18)	0.0577	OC_CMH
M1GA0019802	145.2 (14)	PTPRE	144.3 (14)	0.0845	OC_CMH
ALGA0076446	25.4 (14)	LOC100153845	25.4 (14)	0.0489	OC_CMF
MARC0084086	36.1 (14)	LOC100520051	35.9 (14)	0.0526	OC_CMF
MARC0098684	36.1 (14)	LOC100520051	35.9 (14)	0.0526	OC_CMF
ASGA0062794	38.2 (14)	TBX5	38.1 (14)	0.0780	OC_CMF
MARC0093124	36.9 (14)	MED13L	36.3 (14)	0.0884	OC_CMF
MARC0002724	36.4 (14)	MED13L	36.3 (14)	0.0884	OC_CMF
ASGA0062813	38.4 (14)	RBM19	38.4 (14)	0.0917	OC_CMF
MARC0069757	83.5 (5)	NDUFA12 TMCC3	83.5 (5) 83.6 (5)	0.0917, 0.0891, 0.0037	OC_CMF, OC_DEU, OC_cat
ASGA0062910	39.3 (14)	RPH3A	39.1 (14)	0.0917	OC_CMF
MARC0086568	39.7 (14)	ERP29	39.7 (14)	0.0929	OC_CMF
ASGA0060524	3.4 (14)	SPTLC1	3.4 (14)	0.0449 , 0.0782	OC_DEU, OC_sum
ALGA0105690	2.4 (18)	DPP6	2.4 (18)	0.0823, 0.0287	OC_DEU, OC_sum
H3GA0043430	145.9 (14)	MGMT	145.7 (14)	0.0686	OC_sum
MARC0112236	121.6 (15)	LOC100514990	121.5 (15)	0.0686	OC_sum
ASGA0083649	11.7 (10)	BROX	11.6 (10)	0.0231	OC_cat
ALGA0057003	9.9 (10)	MARK1	10.0 (10)	0.0860	OC_sum
M1GA0007707	16.7 (5)	KRT8	16.7 (5)	0.0100	OC_cat

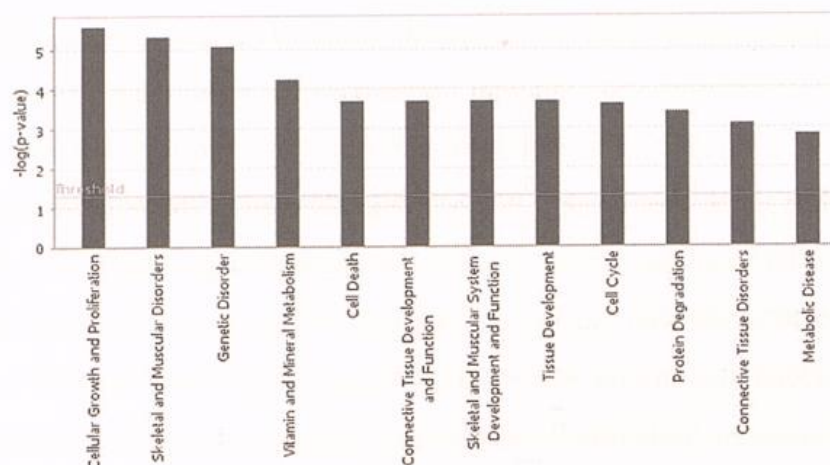


Fig 1. Ingenuity analysis of biofunction enriched for differentially genes expressed.

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