Antepartal insulin-like growth factor concentrations indicating differences in the metabolic adaptive capacity of dairy cows

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Abstract: Cows with different Insulin-like Growth Factor-I (IGF-I) concentrations showed comparable expression levels of hepatic growth hormone receptor (GHR). Suppressor of cytokine signaling 2 (SOCS2), could be responsible for additional inhibition of the GHR signal cascade. The aims were to monitor cows with high or low antepartal IGF-I concentrations ($IGF-I_{\text{high}}$ or $IGF-I_{\text{low}}$), evaluate the interrelationships of endocrine endpoints, and measure hepatic SOCS2 expression. Dairy cows ($n=20$) were selected (240 to 254 days after artificial insemination (AI)). Blood samples were drawn daily (day -17 until calving) and IGF-I, GH, insulin, thyroid hormones, estradiol, and progesterone concentrations were measured. Liver biopsies were taken (day 264 ± 1 after AI and postpartum) to measure mRNA expression (IGF-I, IGFBP-2, IGFBP-3, IGFBP-4, acid labile subunit (ALS), SOCS2, deiodinase1, GHR1A). IGF-I concentrations in the two groups were different ($p<0.0001$). However, GH concentrations and GHR1A mRNA expression were comparable ($p>0.05$). Thyroxine levels and ALS expression were higher in the IGF-I$_{\text{high}}$ cows compared to IGF-I$_{\text{low}}$ cows. Estradiol concentration tended to be greater in the IGF-I$_{\text{low}}$ group ($p=0.06$). It was hypothesized that low IGF-I levels are associated with enhanced SOCS2 expression although this could not be decisively confirmed by the present study.

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Abstract
Cows with different IGF-I concentrations showed comparable hepatic growth hormone receptor (GHR) expression. Suppressor of cytokine signaling 2 (SOCS2), a negative regulator of the GHR signal transduction could be responsible for additional blocking of the GHR. The aims were to monitor cows with high vs. low ante partal IGF-I concentrations (groups: IGF-I\textsuperscript{high} vs. IGF-I\textsuperscript{low}) and model interrelationships of endocrine endpoints and to measure hepatic SOCS2 expression. Dairy cows (n = 20) were selected on day 240 to 254 after AI. Daily (day -17 until calving) blood samples were drawn and analyzed for IGF-I, GH, insulin, thyroid hormones, estradiol and progesterone. Liver biopsies were taken (d 264 ± 1 after AI and postpartum) to determine mRNA abundance (IGF-I, IGFBP-3,-4,-2, acid labile subunit, SOCS2, deiodinase1, GHR1A). The IGF-I concentration remained different between both groups (p < 0.0001). However, GH concentrations and GHR1A expression were comparable (p > 0.05). Thyroxine levels and hepatic mRNA expression of acid labile subunit were higher in IGF-I\textsuperscript{high} compared to IGF-I\textsuperscript{low} cows. Estradiol tended to be higher in the IGF-I\textsuperscript{low} group (p = 0.06). The hypothesis that low IGF-I levels are associated with enhanced SOCS2 expression could not be confirmed by the present study.

Key words: insulin-like growth factor I, growth hormone, cattle, suppressor of cytokine signaling, metabolism
Introduction

The somatotropic axis is a key metabolic pathway during the transition from late pregnancy to early lactation in dairy cows [1]. This endocrine axis is consistent of pituitary derived growth hormone (GH), which is released in a pulsatile fashion by the control of two hypothalamic hormones (growth hormone releasing hormone and growth hormone inhibiting hormone). The liver expresses the highest amount of GH receptors (GHR) and binding of GH to its receptor initiated the production of insulin-like growth factor I (IGF-I) [2-4]. During the peripartal period liver GHR expression decreases in dairy cows and the hepatic IGF-I production decreased as well while GH concentrations increased due to a reduced negative feedback [2,5]. It is well described that the GHR transcript GHR1A decreased towards parturition [2] and even the binding of growth hormone at the hepatic GHR is diminished [6]. Alterations of the GH-IGF-I system were already associated with increased occurrence of postpartum metabolic diseases [7,8] and fertility problems [9]. Therefore basic knowledge of factors or pathways affecting the uncoupling of the somatotropic axis is crucial to consider new management strategies or prophylactic therapies for high yielding dairy cows in the transition period. It was previously supposed that a reduction in feed intake occurring within the last three weeks of pregnancy is not, or not alone responsible for a GHR1A mRNA decrease in dairy cows [10]. In a previous study cows were fed comparable and highly different antepartal IGF-I concentrations were found in cows from one large scale dairy farm [5,7]. These results underpin the idea that additional factors, e.g. at the post-GHR level may block IGF-I production. Increasing estradiol concentrations were suspected as a potential mechanism for attenuation of the GHR signal transduction pathway by induction of suppressor of cytokine signaling 2 (SOCS2) expression [10], which is known as a negative regulator of the GHR signal transduction pathway. Only a few studies addressed the SOCS2 expression in cattle in the transition period. It was demonstrated that limited fed cows with higher estradiol concentrations showed elevated hepatic SOCS2 expression [10] and cows selected for low
IGF-I concentrations in late pregnancy had comparable GHR1A expression and expressed higher (statistical tendency) SOCS2 mRNA [5]. Therefore, the aims of the present study were to monitor cows selected based on their ante partal IGF-I concentration until a spontaneous calving, and model the interrelationships of endocrine endpoints by using a structural equation model. Moreover, it was whether SOCS2 mRNA expression differs between the two IGF-I groups, and this correlates with a physiological increase of estradiol.

Materials and Methods

Cows and selection criteria on the farm

From a large-scale dairy farm (1,100 cows) in Wöpel/Siedenlangenbeck, Saxony-Anhalt, Germany, pluriparous German Holstein Friesian cows in the second to third lactation that had already dried off were used in this study (approved by German legislation on animal welfare Lower Saxony Federal State Office for Consumer Protection 279 and Food Safety, AZ 33.9-42502-04-09/1696). At the dairy farm, cows were housed all-season in a free-stall barn with rubber mats and fed automatically by a band-conveyor system with a total mixed ration twice daily and were provided with a mineral supply (Deutsche Vilomix Tierernährung GmbH, Neuenkirchen-Vörden, Germany). They had free access to water.

In total 158 pregnant cows between 240 and 255 d after AI were clinically examined at the farm by a veterinarian (assessment of posture, locomotion and general wellbeing). Blood samples were collected (10 mL, coccygeal vessels) from clinically healthy cows. An aliquot of 3 mL of blood was immediately subjected to a glutaraldehyde test to assess the immunoglobulin status, as a rapid estimation of overall health [11]. Cows with a positive test result in \( \leq 3 \text{ min} \) were excluded. From animals with a negative glutaraldehyde test, blood samples were collected in EDTA-containing tubes and serum tubes. Serum samples were kept at room temperature (30-60 min) for clotting of the whole blood, and subsequently centrifuged (2,000 x g, 15 min) on the farm within 120 min after blood sampling. Plasma and
serum were stored on ice for the transport and afterwards at -20°C until analysis. The plasma IGF-I concentrations were measured immediately by use of a previously described method and the selection of cows followed a previously established protocol [5]. Due to logistical reasons two cows were selected at each farm visit as previously reported [5]. Selected cows were then transported to the Clinic for Cattle at d 265 after AI at the latest. Based on the IGF-I concentration, in total 20 cows were selected [IGF-I$_{low}$ (n = 10) and IGF-I$_{high}$ (n = 10)]. Here the cows were housed in tie stalls with straw bedding. Cows were fed a diet based on ad libitum access to hay and were additionally fed twice daily 6 kg of corn silage and 1 kg of concentrate (18% CP, St.Mv.18 III Pell.; ForFarmers Bela GmbH, Vechta, Germany). Water was available ad libitum. The cows were clinically examined daily (body temperature measurement, heart rate, breathing rate, rumen filling, food intake). A spontaneous calving was awaited; therefore the cows were examined daily and daily progesterone (P4) was measured. By the time P4 decreased to < 2 ng/mL, the cows were intensely monitored using a web-cam fixed at the stall ceiling.

**Blood sampling**

From d 266 after AI until the day of parturition (day -1), blood samples were collected once daily in the morning from the coccygeal vein into serum tubes and tubes containing potassium-EDTA as an anticoagulant (Sarstedt, Nümbrecht, Germany). Blood samples were collected 30 min after parturition (day 0). EDTA and serum samples were centrifuged at room temperature (2,000 x g, 15 min) within 30 min after sampling and stored at -20°C until analyzed. The blood samples were ordered retrospectively by the day of calving and the day of selection was defined as day -34 (median of all animals; min = day -27 and max = day -43).

**Liver biopsies**

Liver biopsies were taken on d 264 ± 1 after AI (ante partum) and post partum after a physiological release of the fetal membranes (3 – 9 h after the spontaneous calving) to determine mRNA abundance for specific genes (Tab. 1). The biopsies were obtained as
previously described [5]. Approximately 100 mg of liver tissue was obtained and directly transferred into a sterile Eppendorf cup that was immediately frozen in liquid nitrogen and stored at -80°C until mRNA extraction and PCR analysis.

**Hormone and metabolite measurements**

**IGF-I and GH** Total plasma IGF-I concentration was determined using a commercial IGF-I-ELISA (Active IGF-I ELISA; Beckman Coulter, CA, USA) by standard operation manual. The analytic sensitivity was 0.03 ng/mL. The intra- and interassay CV were 3.5% and 8.5%, respectively. Bovine GH concentration was determined using an ELISA as previously described [9,12] with modifications described by Piechotta et al. [5]. The GH concentrations were calculated using Magellan software and cubic spline modus was used (Magellan 3.11, Dortmund, Germany). Intra- and interassay CV were 9.8 % and 12.6 %, respectively. The lowest detection limit for GH was 2.0 ng/mL.

**Thyroxine, Triiodothyronine, Insulin, Progesterone, 17β-Estradiol and Non-esterified fatty acids** Serum thyroxine (T4) and triiodothyronine (T3) concentrations were determined every second day relative to the day of parturition using competitive chemiluminescence immunoassays (Thyroxin LKCT5 and LKT35, respectively; Immulite™ 1000 System, Siemens Diagnostics, USA). Plasma insulin was measured every second day using a RIA (DSL-1600, Diagnostic Systems Laboratories, Inc., Texas, USA). Serum P4 concentrations were determined daily using an automated competitive chemiluminescence immunoassay (LKPG1, Immulite™ 1000 System, Siemens Diagnostics, USA). Serum 17β-estradiol was quantified every second day using a coat-a-count RIA according to the manufacturer’s instructions (Estradiol Coat-a-Count, TKE21, Siemens Medical Diagnostics, CA, USA). Serum concentrations of NEFA were measured at selection on the farm and on day 266 after AI, as well as at day 0 using a photometric automatic clinical chemistry analyzer (ABX Pentra 400, Horiba, Montpellier, France). The test specifications were previously described [5].
RNA isolation and real-time PCR

Total mRNA (0.5 µg total RNA was used for the quantitative PCR [qPCR]) was extracted using the RNeasy Mini Kit for animal tissue and cells (Qiagen, Mississauga, ON, Canada) with QIAcube (Qiagen) according to the manufacturer’s instructions. The quality and integrity of the extracted RNA was assessed using a RNA 6000 Nanoassay for an Agilent 2100 Bioanalyzer (Agilent Technologies, Deutschland, Böblingen, Germany), and the RNA relative integrity number (RIN) was at least > 7.5. The relative abundance of mRNA in the liver biopsy specimen for the qPCR was measured using a BioRAD iQ™5 (BioRad, Munich, Germany). The PCR reaction mix contained 10 µl MESA GREEN qPCR MasterMix Plus for SYBR Assay (Eurogentec, Cologne, Germany), and 0.2 µM of each forward and reverse primer (Eurofins MWG Operon, Ebersberg, Germany) for genes of interest. The PCR-Cycler (Biometra, Göttingen, Germany) was programmed using the following protocol: RNA denaturation at 95°C for 15 min, followed by 43 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s for the amplification phase. Transcripts were visualized using SYBR Green. The procedure to characterize a melting curve for verification of the PCR fragments initiated at 55°C, and the temperature was increased by 0.5°C every 10 s up to a final temperature of 95°C. Relative abundance of mRNA was determined relative to two housekeeping genes (RPS9 and GAPDH) which did not differ between cows with IGF-I\text{low} vs. IGF-I\text{high} (p > 0.05; Tab. 1).

Statistical Analyses

For statistical analyses, SAS (version 9.2., SAS Inc., Cary, NC, USA, S.A.S. 2008) was used. The data were tested for normal distribution using the Kolmogorov-Smirnov test (n > 90; Proc Univariate normal plot) and the Shapiro-Wilk test (n < 90). The data that did not deviate significantly from a normal distribution were presented as the mean ± standard error (SE) or the least-squares means (ls-means) ± SE. All data that were not normally distributed was mathematically transformed. Thus, to achieve a normal distribution before parametric
statistical methods were applied, the following transformations were used: relative abundance of GHR1A, diodinase1 (DIO1) and acid labile subunit (ALS), as well as GH, IGF-I, T4, T3, insulin, NEFA, and E2 blood concentrations were transformed using the logarithmic natural transformation (logX = log(X)). IGF-I- mRNA values were transformed using the arctangent procedure (atanX = atan(sqrt(X*100)). SOCS2 and IGFBP4 values were transformed using the arctangent procedure as well [atanX = atan(SQRT(X))]. IGF-I (d -17 to 0 calving) values were transformed using the square-root procedure (sqrtX = sqrt(X). To examine differences between groups (IGF-I\textsuperscript{low} and IGF-I\textsuperscript{high}) and time a Student’s \textit{t}-tests (Proc ttest) and ANOVA were performed. In not normally distributed data the statistical analyses were done using a Kruskal-Wallis test (Proc npar1way). The mRNA data and NEFA plasma concentrations between d -17 and day of calving (day 0) that were normally distributed were analyzed using an ANOVA (Proc GLM with options lsmeans/pdiff,). The normally distributed data were tested for significant differences using a repeated measures ANOVA with fixed effects using a mixed model procedure (Proc Mixed and method REML). As the main effects IGF-I group (treatment: IGF-I\textsuperscript{low} and IGF-I\textsuperscript{high}), time (d -17 to 0 calving) and interactions (treatment IGF-I × time) were defined. Additionally, the effects of the IGF-I group on IGF-I, GH, T3, T4, P4, E2 and insulin concentrations were tested [13-16]. The Bayesian-Schwarz criterion and Akaike information criterion were used to determine the optimal covariance structure matrix (sqrtIGF-I and T3: spatial power with random effect; logGH: spatial power; logT4: variance components simple structure; logInsulin and P4: first-order autoregressive with random effect and logE2: first-order autoregressive) to use in the statistical model [13,14]. Due to the unbalanced number of individual data points per treatment, the option Satterthwaite was applied to obtain corrected lsmeans. To detect statistical differences between variables, orthogonal contrast analysis was performed. The univariate procedure was used to assess the residuals for normality, and the Levene test was used to test for homoscedasticity. The relationships between and within parameters were evaluated using the Spearman and Pearson
correlation (Proc corr spearman pearson). To control for unequal sample size, the homogeneity of variance and random effect were first evaluated using a Brown and Forsythe test (Proc GLM with option means hovtest = BF) and then a Durbin-Watson test (Proc REG options dw). Obvious outliers (n= 5) were removed before performing the final analysis. The prediction equation was determined by multiple regression analysis using PROC REG with option selection = BACKWARD. For all procedures, the statistical significance was pre-established at p< 0.05. p-values of between p > 0.05 and p < 0.10 were considered as statistical tendencies.

A structural equation model was calculated by using the programs AMOS (version 18.0, 2009) [2] and PROC CALIS of SAS (SAS version 9.2 2010). This model was calculated to estimate causal relationships among variables using a combination of statistical data and qualitative causal assumptions [17-19]. The mayor indices showed a poor fit, indicating that the model structure was not acceptable. However, centrality and CN showed a good fit. In general, of the 18 fit indices those that were evaluated, 14 supported acceptance of the presented model.

Results

Cows

In total 158 cows between 240 and 254 d after AI were examined on the farm, and in 151 cows blood samples for IGF-I measurement were drawn. The mean IGF-I concentration was 161.4 ± 65.7 ng/mL, the minimum value was 46.3 ng/mL, and the maximum value 450.0 ng/mL. The lower 25% quartile, median, and upper 75% quartile were 109.9, 153.5, and 202.3 ng/mL, respectively. The 20 selected cows had comparable body weights (IGF-I\textsubscript{low} = 661 ± 65 vs. IGF-I\textsubscript{high} = 607 ± 65 kg) and comparable milk yields in the previous lactation (IGF-I\textsubscript{low} = 7743 ± 1265 vs. IGF-I\textsubscript{high} = 7045 ± 607 kg/305days). One of the 10 IGF-I low cows transported from the farm to the clinic was excluded from the study due to highly
aggressive behavior. Spontaneous calving was monitored in 19 cows, and obstetrical help (two persons) was needed in only 1 cow.

**Hormone concentrations at day of selection** At day of selection, the cows had highly different IGF-I plasma concentrations, whereas growth hormone was comparable. The NEFA concentrations tended to be higher in cows with low IGF-I concentrations ($p = 0.053$) compared to IGF-$I_{\text{high}}$ counterparts. The other tested metabolic hormones (insulin and thyroid hormones) as well as sexual steroid hormones were comparable between IGF-$I_{\text{high}}$ and IGF-$I_{\text{low}}$ (Tab. 2).

**IGF-I and Growth Hormone** Cows grouped in IGF-$I_{\text{high}}$ and IGF-$I_{\text{low}}$, based on IGF-I levels on day 240 – 254 post AI (27 – 43 days before calving), still showed significantly different IGF-I concentrations during the 17 d before calving ($p = 0.0063$).

The IGF-I concentrations decreased from day -17 until the day of calving significantly in both groups. Plasma growth hormone significantly increased towards the spontaneous calving in both IGF-I groups and were comparable at day of selection and during the 17 d before calving. (Fig. 1).

**Thyroid Hormones** The thyroxine concentrations decreased towards calving in both IGF-I groups, and were significantly higher in cows with high IGF-I concentrations. The concentration of triiodothyronine remained relatively constant and similar between pluriparous cows selected for IGF-$I_{\text{high}}$ and IGF-$I_{\text{low}}$ (Fig. 2.).

**Insulin** A significant decrease of insulin concentration was observed in both IGF-$I_{\text{high}}$ versus IGF-$I_{\text{low}}$. There was a continuous decrease in the cows of group IGF-$I_{\text{low}}$ between day -17 and parturition (d0), whereas in cows out of group IGF-$I_{\text{high}}$ insulin remained on a constant level until two days before the calving (day-3), and showed a sharp decrease between the day of parturition and when samples were taken 30 min after parturition (d0) (Fig. 2.).

**Sexual steroid hormones** The $17\beta$Estradiol concentration increased significantly from day -17 ($\log_{\text{Estradiol}}: IGF-I_{\text{low}} = 4.5 \pm 0.2$ vs. IGF-$I_{\text{high}} = 4.2 \pm 0.2$ pg/mL) to the day of calving
(log\text{Estradiol}: \text{IGF-I}^{\text{low}} = 6.8 \pm 0.2 \text{ vs. } \text{IGF-I}^{\text{high}} = 6.4 \pm 0.2 \text{ pg/mL}) in both \text{IGF-I}^{\text{high}} \text{ and } \text{IGF-I}^{\text{low}} (p < 0.0001). Cows in the low \text{IGF-I} \text{ group tended to show (}p = 0.06\text{) higher 17\beta\text{Estradiol concentration (log\text{Estradiol}: 5.6 \pm 0.2 \text{ pg/mL}) compared to } \text{IGF-I}^{\text{high}} (\text{log\text{Estradiol}: 5.2 \pm 0.1 pg/mL}). \text{Progesterone significantly decreased towards calving in both groups (}p < 0.0001\text{) with comparable concentrations between IGF-I groups (}\text{IGF-I}^{\text{low}} = 4.9 \pm 0.4 \text{ vs. } \text{IGF-I}^{\text{high}} = 4.8 \pm 0.4 \text{ ng/mL}; \: p > 0.05\text{).}

Non esterified fatty acids The mean NEFA concentration 266 days after AI was significantly higher in cows with low IGF-I concentrations (1147 \pm 221 \text{ mMol/L}) compared to cows with high IGF-I levels (567 \pm 118 \text{ mMol/L}; p < 0.01). On the day of calving, the same relationship existed. Cows with low IGF-I levels had significantly higher NEFA concentrations (1417 \pm 207 \text{ mMol/L}) compared to cows with high IGF-I levels (1219 \pm 137 \text{ mMol/L}; p < 0.05). In both groups, NEFA concentrations significantly increased towards calving (p < 0.01).

Interrelationship between tested endocrine variables- Structural equation model-
The standardized pathway diagram had 8 parameters to be estimated with 15 regression weights, 1 covariance (correlations), and 9 variances that were statistically significant (Fig. 3).

Hepatic gene expression
Ante partum, the cows of the group IGF-I^{\text{high}} expressed significantly more IGF-I mRNA compared to cows with low IGF-I concentrations. Interestingly, the GHR1A mRNA expression was comparable between IGF-I^{\text{high}} and IGF-I^{\text{low}}. The GHR1A mRNA expression significantly decreased towards parturition by the same magnitude in IGF-I^{\text{high}} and IGF-I^{\text{low}}. Neither IGF-I group nor time had an influence on the SOCS2 expression. The relative abundance of liver DIO-1 was significantly higher ante partum than post partum. Cows of group IGF-I^{\text{high}} had a higher expression of DIO1 ante partum and postpartum compared to
cows with low IGF-I concentrations hepatic expression of IGFBP3, and acid labile subunit decreased significantly towards parturition in both IGF-I groups (Tab. 3).

Interaction between IGF-I group, Estradiol and SOCS2 expression

The IGF-I level neither was significantly associated with the ante partal, nor with the post partal, estradiol concentration or the increase of estradiol before calving (diffE2, P>0.05). The SOCS2 expression was also not associated with the IGF-I plasma level (IGF-I$^{\text{high}}$ vs. IGF-I$^{\text{low}}$).

Discussion

Twenty cows were selected and daily blood sampling revealed that IGF-I concentrations remained significantly different until the day of calving. In agreement to the conclusion of Kessel et al. [20], a selection of cows with an individual variation of the adaptive ability in the dried off period was possible. The authors concluded that cows with higher ketone body concentration postpartum were less able to cope with metabolic stress than cows in normoketonaemic conditions. It was previously suggested that the measurement of metabolic endpoints, like β-hydroxybutyrate, seemed to be more efficient and sensitive as selection criteria for metabolic adaptive capacity compared to the mathematical calculation of cows energy balance or measuring feed intake or weight loss in cows [21]. The cows of the group IGF-I$^{\text{low}}$ displaced higher NEFA concentrations compared to IGF-I$^{\text{high}}$, only in statistical tendency. This result lead to the assumption that cows with markedly lower IGF-I levels were in a metabolic status of enhanced lipolysis irrespective of food offer and housing. This result indicate that the IGF-I concentration also seemed to be suitable as metabolic marker for adaptive capacity. However, the factors that may causally determine the differences between the selected cows is not clear so far. Insulin-like growth factor-I, as well as the major binding proteins (IGFBP3 and ALS), are produced in the liver under growth hormone control. However, neither hepatic GHR1A expression nor blood GH concentrations were different between IGF-I$^{\text{high}}$ and IGF-I$^{\text{low}}$. Similar results were reported by Kobayashi et al. [22] in
lactating dairy cows (153-265 days in lactation) fed either restricted or non-restricted diets showed different IGF-I concentration with comparable GHR1A levels. In late pregnancy however, the GHR1A transcript decreased, as also detected in the present study in both IGF-I groups. Energy deficiency and ghrelin concentrations were shown to affect the GH peak concentration after a re-feeding following a starvation phase in early lactation, but not in late pregnancy suggesting that the GH-IGF-I axis might be completely differently regulated during late pregnancy and early lactation [23]. Moreover, the cows in the present study were fed and housed under comparable conditions, suggesting that additional factors regulate the total IGF-I and binding protein concentration ante partum.

The structural equation model revealed a significant direct effect of time on the IGF-I concentration, but no other tested hormones seem to influence the IGF-I concentration directly. However, several direct effects of the IGF-I hormone level on other hormones were detectable. The IGF-I group directly influenced T4, insulin, and estradiol levels. It was hypothesized that the selected cows differed in their metabolic adaptive capacity. This fact seemed to be further confirmed by the direct effect of the IGF-I groups on T4. An interaction between the GH-IGF-I System and thyroid hormone status is known [5,24] but less is known about thyroid hormones and their influence on metabolic adaption in late pregnancy. The decrease of T4 detected in the present study is in accordance to literature [25]. Also a decrease in T3, described as low T3 syndrome, was detected in early lactation versus late pregnancy, and is suspected to arise from a phase of chronic catabolism [25,26]. Cows with high IGF-I concentrations showed significantly higher T4 concentration, whereas T3 and liver DIO1 mRNA expression remained comparable between both groups. Liver DIO1 expression was significantly higher ante partum compared to immediately postpartum in both IGF-I groups, which goes along with the decreasing T4 concentration, as DIO1 converts T4 to T3. Short-term changes within the thyroid status of cattle had no major impact on the GH-IGF-I axis [4], but to our knowledge less is known about the impact on differences within the somatotropic
axis and thyroid hormones. The structural equation model suggested that the IGF-I concentration, but also E2 and T3, directly and TIME indirectly affect the T4 concentration and that these parameters might explain 75% of the variance in T4. Therefore, the differences in T4 concentrations, might follow a diverging adaption of the GH-IGF-I system. This idea might also be substantiated by the fact that the thyroid hormone concentration was comparable at time of selection and started to differ approximately 10 days before calving. Interestingly, T4 had a direct, although low, effect on the GH concentration which itself directly affects the T3 concentration.

Growth hormone directly acts on the adipose tissue and enhanced lipolysis [27]. However, no difference in GH concentration or GHR1A RNA expression in the liver was detectable between both animal groups. As the somatotropic axis uncouples a decrease in GHR1A mRNA transcript, and a subsequent decrease in IGF-I concentrations, is obvious which induces an increase in GH concentrations [2,6]. Hepatic GHR1A mRNA expression and IGF-I concentration decreased in both IGF-I groups. However, cows with high IGF-I concentrations showed a significantly higher IGF-I mRNA expression ante partum, but the GHR1A transcript was comparable between both groups of cows. In rats and humans, it is known that the somatotropic axis is differentially affected by either nutrient deficiency or protein restriction [28]. It was suggested that protein restriction causes post GHR resistance, and that the low IGF-I concentrations are not mediated by reduced GH binding compared to general feed restriction in which IGF-I mRNA and IGF-I blood concentrations decreased [3,28]. However, the cows of the 2 IGF-I groups were fed comparable diets. In general, it might be more difficult to differentiate between protein or feed restriction in cows due to ruminal fermentation, and data on influences of nutrient intake during the peripartal time on the IGF-I concentration are conflicting [29,31]. However, the correlation between low IGF-I mRNA expression, and comparable GHR1A mRNA, and GH lead to the hypothesis that the post receptor signaling might be negatively regulated in cows with low IGF-I concentrations.
[5,22]. Oral estrogens, for instance, reduced IGF-I level in women [32], and a connection between estrogen concentration and the attenuation of the post GH receptor pathways through expression of SOCS2 was previously described [33,34]. No difference in the SOCS2 expression was detectable between cows selected for IGF-I differences, and also in ante- and postpartum no differences in relative abundance of SOCS2 were obvious. This result is in conflict with the study of Winkelman et al. [10], in which SOCS2 mRNA expression increased towards calving. However, the most pronounced increase of SOCS2 was detected between day – 7 and 2 days after parturition in the study of Winkelman et al. study, whereas in present study SOCS2 expression was measured earlier before calving and immediately after calving [10]. Therefore, the missing increase in SOCS2 could be due to differing time points of liver biopsy sampling. In conclusion, cows selected antepartal during the dry period for IGF-I^{high} versus IGF-I^{low} concentrations, showed differences within the thyroid hormone metabolism, and cows with low IGF-I concentrations expressed low ALS mRNA. This binding protein affects the half-life of IGF-I and might be responsible for low total IGF-I concentrations. Even if the expression of ALS is also growth hormone dependent, growth hormones concentrations and GHR1A transcription were comparable between IGF-I^{high} and IGF-I^{low} groups. Estradiol concentrations were higher in cows with low IGF-I concentrations. However, a higher expression of SOCS2 as a negative regulator of the GHR signal transduction could not be confirmed by the present study. In conclusion the present data indicated several endocrine factors differing between IGF-I^{high} versus IGF-I^{low} cows, however the hypothesis that low IGF-I levels might be associated with enhanced SOCS2 expression could not be confirmed by the present study, although E2 concentrations in the prepartal period differed slightly between cows. However, E2 decreases rapidly after parturition and might already be comparable to the sample taken on d 264 after AI. If the SOCS 2-expression occurs fast, this study might have missed the exact time. Therefore, further studies are warranted to test if SOCS 2 expressions block the GHR signal transduction in the liver of late
pregnant dairy cows. As low antepartal IGF-I concentrations were already associated with increased occurrence of postpartal metabolic diseases and the data of the present study clearly demonstrated that IGF-I^low^ cows showed a higher lipolysis rate indicated by NEFA levels those cows should be managed separately compared to cows with high IGF-I levels.

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Table Legends

Table 1. Real-time PCR primers for genes of interest in liver biopsy specimens of late-pregnant cows obtained on day 264 ± 1 after AI and within 30 minutes after a spontaneous calving (day 0).

Table 2. Concentrations (mean ± standard error) of endocrine [IGF-I, log = logarithmic transformed, growth hormone, triiodothyronine (T3), thyroxine (T4), insulin (logInsulin), 17β-estradiol (logE2), progesterone (P4)] and metabolic endpoints [non-esterified fatty acids (logNEFA))] in cows selected for IGF-I-low vs. IGF-I-high plasma concentrations at day of selection on a dairy farm (240 – 254 days after AI). Statistical significant differences between columns were indicated by using different letters (a,b;p< 0.0001). A statistical tendency between IGF-I high and IGF-I low cows were indicated by (*p = 0.053). Values in square brackets are not transformed.

Table 3. Liver biopsy mRNA expression. The data are expressed as mean ± standard errors. The data are expressed as the mean±standard errors. Significantly differences between rows were indicated by different letters. A statistical tendency was indicated by * (p = 0.0582)
**Figure Legends**

Figure 1. Insulin-like Growth Factor (IGF-I) and growth hormone (GH) plasma concentrations in cows previously selected based on their IGF-I concentration in a group with IGF-I low vs. IGF-I high concentrations. The IGF-I values are square-root transformed (sqrtIGF-I). GH values are logarithmic transformed (logGH). The table shows results of the mixed-model ANOVA with a covariance structure matrix spatial power with the random effect (SP(POW)+RE). Data are displayed as the last square means ± standard error. BIC = Bayesian-Schwarz criterion. AIC = Akaike information criterion.

Figure 2. Thyroxine and Triiodothyronine serum concentrations in cows previously selected based on their IGF-I concentration in a group with IGF-I low vs. IGF-I high concentrations. The thyroxine values are logarithmic transformed (logThyroxine). The table shows results of the mixed-model ANOVA with a covariance structure matrix spatial power with the random effect (SP(POW)+RE). Data are displayed as the last square means ± standard error. Significant differences between IGF-I groups at individual time points were indicated with a, b (p < 0.05) and a statistical tendency c†, d (P = 0.06). BIC = Bayesian-Schwarz criterion. AIC = Akaike information criterion.

Figure 3. Standardized pathway diagram calculated by using a structural equation modeling of ante partal hormones in pluriparous dairy cows. The standardized path coefficients were depicted over or next to the respective arrow path (p < 0.05). Endogenous dependent variables were represented in a rectangle (LogIGF-I = logarithmic-transformed insulin-like growth factor-I, LogGH = logarithmic-transformed growth hormone, LogT3 = logarithmic transformed triiodothyronine, T4 = thyroxine, LogInsulin = logarithmic-transformed insulin, LogE2 = logarithmic transformed 17β estradiol, P4 = progesterone, HF=Holstein Frisian), and the exogenous independent
variable was represented by a rectangle in mid of the model (time = day -43 to 0 calving) and exogenous variables errors were represented in circles (e = error: eIGF-I = error of IGF-I; eIn = error of insulin; eT3 = error of T3. eP4 = error of P4. eGH = error of GH; eE2 = error of E2. eT4 = error of T4). Double-headed errors denoted these covariances. Model of squared multiple correlations were calculated and R-square values were depicted next to the rectangles of variables.