Journal of Equine Veterinary Science 109 (2022) 103827

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research Papers

Moderate Differences in Plasma Leptin in Mares Have no Effect on Either the Amino Acid or the Fatty Acid Composition of the Uterine Fluid

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ARTICLE INFO

Article history:
Received 7 June 2021
Received in revised form 18 October 2021
Accepted 22 November 2021
Available online 26 November 2021

Keywords:
Horse
Uterine fluid
Metabolic hormones
Body condition, Reproduction

ABSTRACT

Female mammalian reproductive functions are closely linked to body condition and metabolic status. Energy homeostasis is regulated by endocrine hormones such as insulin, IGF-I, leptin, and adiponectin via the hypothalamic-pituitary-adrenal axis. These metabolic hormones and their receptors are also expressed in reproductive tissues and the embryo. We investigated the relationship between circulating leptin and the fatty acid (FA) and amino acid (AA) composition of the equine uterine fluid (UF) and peripheral blood plasma (BP) by using a mass spectrometry-based approach. UF and BP were collected from ten broodmares on days 6 and 7 *post ovulation*, respectively. The mares were retrospectively assigned to two groups according to their BP leptin concentrations (high leptin [> 1.6 ng/mL] versus low leptin [<0.8 ng/mL]). Specific AA and FA compositions for BP and UF were found with different levels of respective metabolite abundances. The main FAs in BP were stearic, palmitic and linoleic acid. In UF, the three most abundant FAs were eicosapentaenoic, arachidonic and stearic acid. The AA profile of BP was dominated by glycine, glutamine, serine and alanine, which were likewise among the highly abundant AAs in UF. In UF, glutamic acid had by far the highest concentration. Therefore, BP leptin concentration within a physiological range does not seem to affect the specific FA nor the AA composition of the UF. The composition of the UF may therefore be mediated by local rather than by peripheral metabolic hormones.

1. Introduction

Reproduction in placental mammals is energetically expensive, resulting in a trade-off between investment in reproduction and maternal maintenance of body condition. Metabolic hormones such as insulin, insulin-like growth factor I (IGF-I), adiponectin and leptin are responsible for energy homeostasis of the body by centrally regulating the formation and use of energy stores via the hypothalamic-pituitary-adrenal axis. The secretion of metabolic hormones is not only triggered directly by metabolic cues, for example energy surplus or deficit [1], but also by environmental factors such as the photoperiod [2]. These mechanisms stimulate fertility and reproduction under favorable conditions and inhibit it in

Conflict of interest statement: The authors state that there is no conflict of interest.

Animal welfare/ethical statement: The authors state that all procedures involving animals in this study have been approved by the responsible ethics committee (“Comite Régional d'Ethique pour l'Expérimentation Animale du Limousin”). The procedures were performed as part of the Foetalim protocol (protocol number 5-2013-5).

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https://doi.org/10.1016/j.jevs.2021.103827

nutritionally poor environments. Metabolic hormones that regulate and reflect the current energy availability are therefore mediators to adapt energy stores for physiological body condition maintenance and surplus reproductive expenses.

Leptin is a proteohormone that is predominantly produced in adipose tissue and released in response to insulin [3], free fatty acid [4] and glucocorticoid [5,6] signaling. By crossing the blood-brain-barrier, in the hypothalamus, leptin upregulates pro-opiomelanocortin neurons, thereby decreasing appetite [7]. Leptin stimulates energy expenditure by increasing glucose and fatty acid (FA) oxidation in muscle cells and adipocytes. In seasonal breeders including the horse, the increase in plasma leptin is involved in mediating the transition from anestrous to ovarian cyclicity, which is interpreted as adaptation to food availability [2,8-10]. While a moderate weight gain after the winter period is considered physiological [11], pronounced obesity in contrast negatively impacts on fertility [16,21]. To improve horse breeding programs, body condition scoring systems (BCS) have therefore been implemented [12]. Fat thickness (FT) was reported to positively correlate with plasma leptin levels and BCS [13–15], while adiponectin negatively correlated to those parameters [16].

Apart from its function as endocrine metabolic hormone, leptin acts in a paracrine manner in reproductive tissues, as evidenced by the detection of leptin receptors in the ovary [17], the oviduct [18], the endometrium [19–21] and the placenta [22–24]. A deficient leptin receptor expression is linked to subfertility in humans. Although the nature of the relationship is not entirely clear [19], leptin may influence the composition of the uterine fluid (UF). To date, the composition of the UF as complex medium is largely unknown. It contains carbohydrates, pyruvate, lactate, proteins, FAs and AAs [25–33]. It derives in part from uterine transudation as well as from endometrial secretions [26,30] and plays a major role in supporting early embryo development by serving as an exchange medium for signaling factors and by providing nutrients to the embryo.

Leptin and its receptors are similar in structure to the interleukin 6 family of cytokines [34] and leptin has been shown to act as a pro-inflammatory mediator [35]. Leptin increases vascular permeability and stimulates angiogenesis in a paracrine and/or autocrine fashion [45]. Enhanced vascular permeability can potentially increase transudation of plasma components such as FAs into the uterine lumen. Furthermore, leptin could also directly impact on the secretory activity of the endometrium via leptin receptors as the latter is expressed in the endometrial glandular epithelium in humans, swine and dogs [19,21,22].

Leptin acting on the endometrium can either be derived from endometrial secretion [36] or from adipocyte secretions via systemic blood as the endometrium is a highly vascularized tissue [37]. The influence of systemic metabolic hormone levels on the local microenvironment of the reproductive tract has not been investigated in detail. However, the fact that plasma leptin levels are reflected in the follicular fluid of mares [38] suggests a comparable direct link between the former and the composition of the uterine fluid.

Furthermore, leptin regulates the functionality of AA transporters in various tissues [39,40] and could therefore influence endometrial AA uptake from the BP and subsequent transport into the uterine lumen. The role of leptin on early embryonic support is denoted by embryonic expression of both leptin and its receptor, as observed in cattle [41] and horses [42]. Indeed, already the early embryo is capable of AA metabolism [43–46]. In the horse, AA availability might be of special importance for nutrient supply during the prolonged preimplantation period.

Additional support for the influence of metabolic endocrine hormones on embryo development is provided by the finding that lipid contents in day 8 embryos were altered in obese compared to normal mares [47]. In these obese mares, BP concentrations of insulin and leptin were elevated, but it remains unclear if the observed effect on embryo lipid content is directly mediated by leptin or caused by other hormonal disbalances in pronounced obesity.

Thus, the mechanisms by which body condition is translated into hormonal signals and in turn affects the uterine milieu in which the embryo develops are manifold and only poorly understood to date [48]. Here, we investigated if BP leptin concentration as a measure of metabolic status correlated with the UF composition regarding the composition of AAs and FAs, in the mare. For this purpose, we collected UF on days 6 and 7 post ovulation, respectively. This corresponds to the timepoint where the embryo would enter the uterus [49].

2. Materials and Methods

2.1. Animals

Ten multiparous, non-pregnant warmblood mares (aged 7–22 years) of the breeding herd at the Station Expérimentale des Haras at Chamberet, France (registration number C1903602) were used for this study during the months of March and April as part of the Foetalim protocol that received ethical approval from the local ethics committee (“Comite ´Régional d’Ethique pour l’Expérimentation Animale du Limousin”) under protocol number 5-2013-5. All broodmares in the study were in good general health condition and reproductively sound as documented by their continuous successful breeding and foaling history and by regular ultrasound examinations in the course of breeding management. Breeding management did not include more invasive genital health examinations such as endometrial swabbing or biopsies.

During the winter period, the barren broodmares were housed in groups in open stables where they had free access to hay, haylage, water, vitamins, and mineral salts. Depending on external factors, such as social status, age, number of previous pregnancies, mares exhibited weight loss or gain to a certain degree over the winter season. In the summer period, the broodmares were kept on pastures where they all (re)gained weight. The procedures described below were part of the routine reproductive management of the mares.

2.2. Cycle Management

In the course of the breeding management of the stud herd, the onset of the first ovarian cycle of the season was assessed by transrectal ultrasound examinations of the reproductive tract beginning in mid-April, prior to letting the animals on pasture. When mares exhibited a follicle >3.5 cm in diameter, they received an intravenous injection of hCG (750 I.U. Chorulon 1500 I.E./mL, MSD Animal Health, Germany) and were subjected to subsequent ultrasound examinations every 24 hours over 2 days after hCG treatment to confirm ovulation (Aloka SSD 500, 5MHz, Aloka-Hitachi Medical Systems, Saint-Priest, France) (Fig. 1). The day of ovulation is referred to as day 0.

2.3. Assessment of Body Condition Score (BCS) and Ultrasonographic Measurement of Subcutaneous Fat Thickness (FT)

The Body Condition Score (BCS) of the broodmares was routinely assessed at the end of the winter period in March. The BCS of the mares selected for the study was additionally recorded on the day of the first ultrasound examination in April. The BCS was determined using the method established by Arnaud et al. [50]. Evaluation parameters included the visual and palpatory assessment of body fat at five defined anatomic sites (neck, withers, tail-

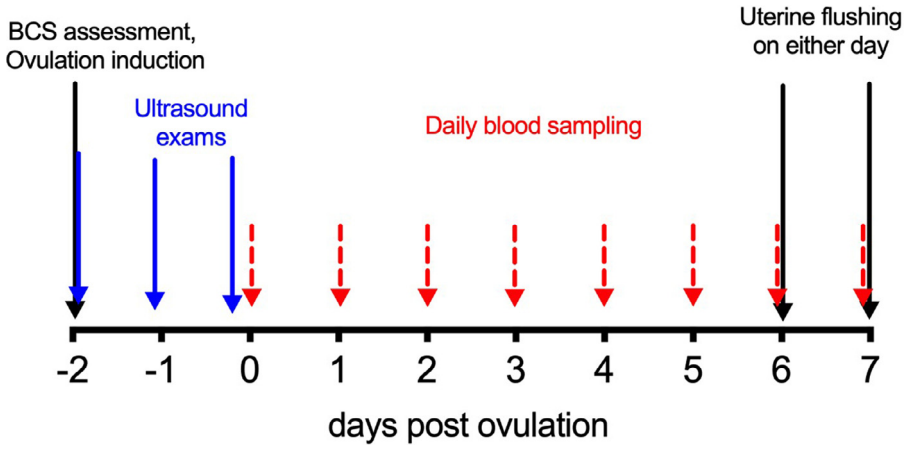

Fig. 1. Scheme of cycle management and sample collection. Body condition score (BCS) was assessed during the routine ultrasound (US) exam mid of April. Mares exhibiting follicles > 3.5 cm were subjected to ovulation induction. The individual date of ovulation was confirmed by subsequent ultrasound exams (blue arrows) every 24 hours until ovulation was confirmed. Daily blood plasma samples (red arrows) were collected from the day of ovulation to the day of uterine fluid collection on day 6 (n = 3) and 7 (n = 7) *post ovulation*, respectively. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

head, ribs, behind the shoulder) on a scoring range from 1 (emaciated/poor) to 5 (obese). Subcutaneous fat thickness (FT) was also evaluated by ultrasound, caudal from the withers between the 10th and 14th rib. BCS and FT were assessed by a single skilled person before determining the BP leptin levels.

2.4. Grouping of Animals

The ten mares were retrospectively assigned to two groups based on their averaged BP leptin levels over seven consecutive days (Low leptin, n = 5 animals and High leptin, n = 5 animals) according to Robles et al. [52]. BP leptin concentrations significantly differed (LMM: χ^2 (1) = 19.156, $P < .001$) between the two groups (0.44 ± 0.04 vs. 2.32 ± 0.10 ng/mL).

2.5. Blood Sampling and Uterine Flushing

Blood samples (10 mL) were taken every morning, 1 to 2 hours after feeding, from the jugular vein starting from the day of ovulation until the day of uterine flushing on days 6 and 7, respectively, *post ovulation* (Fig. 1). Blood samples were collected into tubes containing anti-coagulant (EDTA) and centrifuged at 1500 × g for 10 minutes. Plasma was frozen at -20°C until further analysis. Prior to uterine flushing, the reproductive tract of the mares was examined by ultrasound to exclude pathologic uterine fluid accumulations and to confirm the presence of a corpus luteum.

The uterus was flushed transcervically (embryo flushing catheter, CH28, 90 mm length, Minitueb, Germany) with Ringer solution (Fresenius Kabl AG, Germany) and fluid was retrieved via gravity flow. Due to the difficulty to retrieve enough fluid, the inserted volume ranged from 150 to 300 mL. Inserted and retrieved fluid volumes were recorded. All collected fluid samples were clear. The collected fluid was filtered through a sterile 0.22 µm filter unit (Millex-GS, Millipore, Co. Cork, Ireland), centrifuged (800 × g for 10 min) and frozen at -20°C until further analysis.

2.6. Analysis of Total Protein (TP)

To determine the total protein (TP) content of the UF, the commercial bicinchoninic assay (BCA) (Thermo Fisher Scientific, USA) was used according to the provided protocol. The TP content was used to account for the different inserted flushing volume [51].

2.7. Hormone Analysis

Plasma leptin and adiponectin concentrations were determined using a human leptin and adiponectin AlphaLISA immunoassay kit (PerkinElmer, USA), respectively, according to manufacturer recommendations. Both hormone tests were described and validated by Robles, Nouveau [52]: for leptin, the minimum level of detection was 0.26 ng/mL and intra- and inter-assay coefficients of variation were 8% and 8.3%, respectively. The minimum level of detection for adiponectin was 0.019 ng/ml. Intra- and inter-assay coefficients of variation were 8% and 8.9%, respectively.

Plasma insulin concentrations were measured using a human insulin AlphaLISA immunoassay kit (PerkinElmer, USA) as previously described and validated by Robles et al. [11]: the minimum level of detection was 5.3 mUI/l and intra- and inter-assay coefficients of variation were 6% and 7%, respectively.

IGF-I was analyzed using a commercial immunoradiometric assay (Beckman Coulter IRMA IGF-I, A15729) according to the provided protocol. The assay has previously been validated for the cow [53].

2.8. Analysis of Fatty Acids

The FA profile was determined by gas chromatography mass spectrometry (GC-MS) as described previously [54]. The concentration of the measured FAs was related to the inserted fluid volume [54].

2.9. Analysis of Amino Acids

The quantitative measurements of amino acid concentrations were performed using targeted LC-MS/MS [55]. Plasma (10 µl) and uterine fluid (50 µL) samples were dissolved in 500 µL ice-cold methanol containing an internal standard mixture of 18 labeled amino acids. After centrifugation (10 min, 10°C, 2300 x g), samples were dried. Metabolites were derivatized to their butyl esters [56]. Briefly, a mixture of 95% n-butanol and 5% acetylchloride (v/v) was added to the dried samples. Subsequently, the samples were incubated at 60°C for 15 minutes at 600 rpm (Eppendorf Thermomixer Comfort; Eppendorf, Hamburg, Germany). The samples were dried and reconstituted in a 200 µl mixture of methanol/water/formic acid (70/30/0.1% v/v).

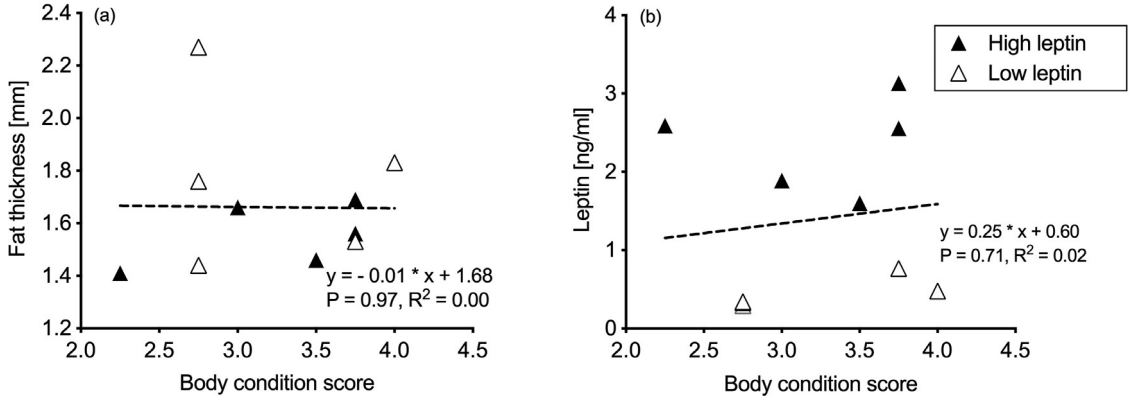

Fig. 2. Correlation of body condition score with (A) fat thickness and (B) plasma leptin concentration. The two groups (High leptin – bold triangle, Low leptin – clear triangle) are indicated. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

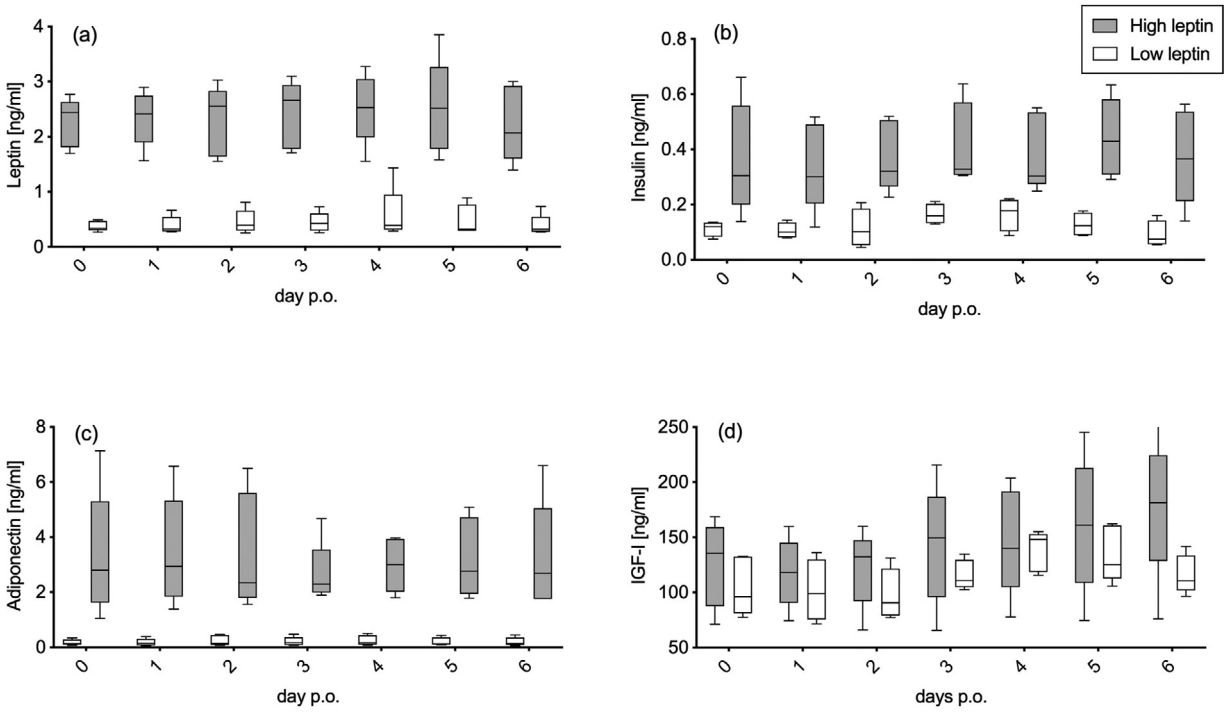

Fig. 3. Box plots of median plasma concentration of metabolic hormones ([A] leptin, [B] insulin, [C] adiponectin, [D] IGF-I) during 7 consecutive days *post ovulation*. The high leptin group displayed higher concentrations of insulin ($P = .004$) and adiponectin ($P < 0.001$) than the low leptin group. Plasma IGF-I concentrations (Fig. 3D) were affected by the interaction between day and group LMM: $\chi^2_1 = 4.145$, $P = 0.042$). Further post-hoc comparisons showed that IGF-I plasma concentration increased in both leptin groups throughout the 7-day sampling period (LMM: leptin high, $\chi^2_1 = 23.360$, $P<0.001$; leptin low, $\chi^2_1 = 9.565$, $P = 0.004$). The different groups are indicated (High leptin - grey box, Low leptin – white box). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

The analysis was performed on a triple quadrupole QTRAP 5500 LC-MS/MS system operating in positive ESI mode (AB Sciex, Framingham, MA) equipped with a 1200 series binary pump (Agilent, Santa Clara, CA) and coupled to an HTC pal autosampler (CTC Analytics, Zwingen, Switzerland). Chromatographic separation was achieved using a Zorbax Eclipse XDB-C18 column (length 150 mm, internal diameter 3.0 mm, particle size 3.5 µm; Agilent). Analytes were measured in scheduled multiple reaction monitoring. For quantification of amino acids, a 10-point calibration was performed, using a mixture containing all amino acids in the measurement (A9906 amino acid standards, Sigma-Aldrich, Taufkirchen, Germany). For quantification of acylcarnitines, area ratios of analyte to internal standard were calculated and multiplied with the internal standard concentration. Data analysis was done using Analyst 1.7.0 software (AB Sciex).

In line with earlier studies [28], the concentration of AAs was related to the TP.

2.10. Statistical Analysis

To analyze the relationship between leptin and BCS, and between FT and BCS, a linear regression analysis (GraphPad PRISM 8, Version 8.3.1, GraphPad Software, LCC) was performed after testing for normality. A linear regression analysis was likewise performed to study the relationships between the metabolic hormone's leptin, insulin, adiponectin and IGF-I. The regressions were carried out on the average hormone concentrations for each individual for each of the seven consecutive samples collected prior to uterine flushing.

To test for differences between groups in the FA and AA composition, we used Mann-Whitney-U test (SPSS, Version 26.0.0.0, IBM

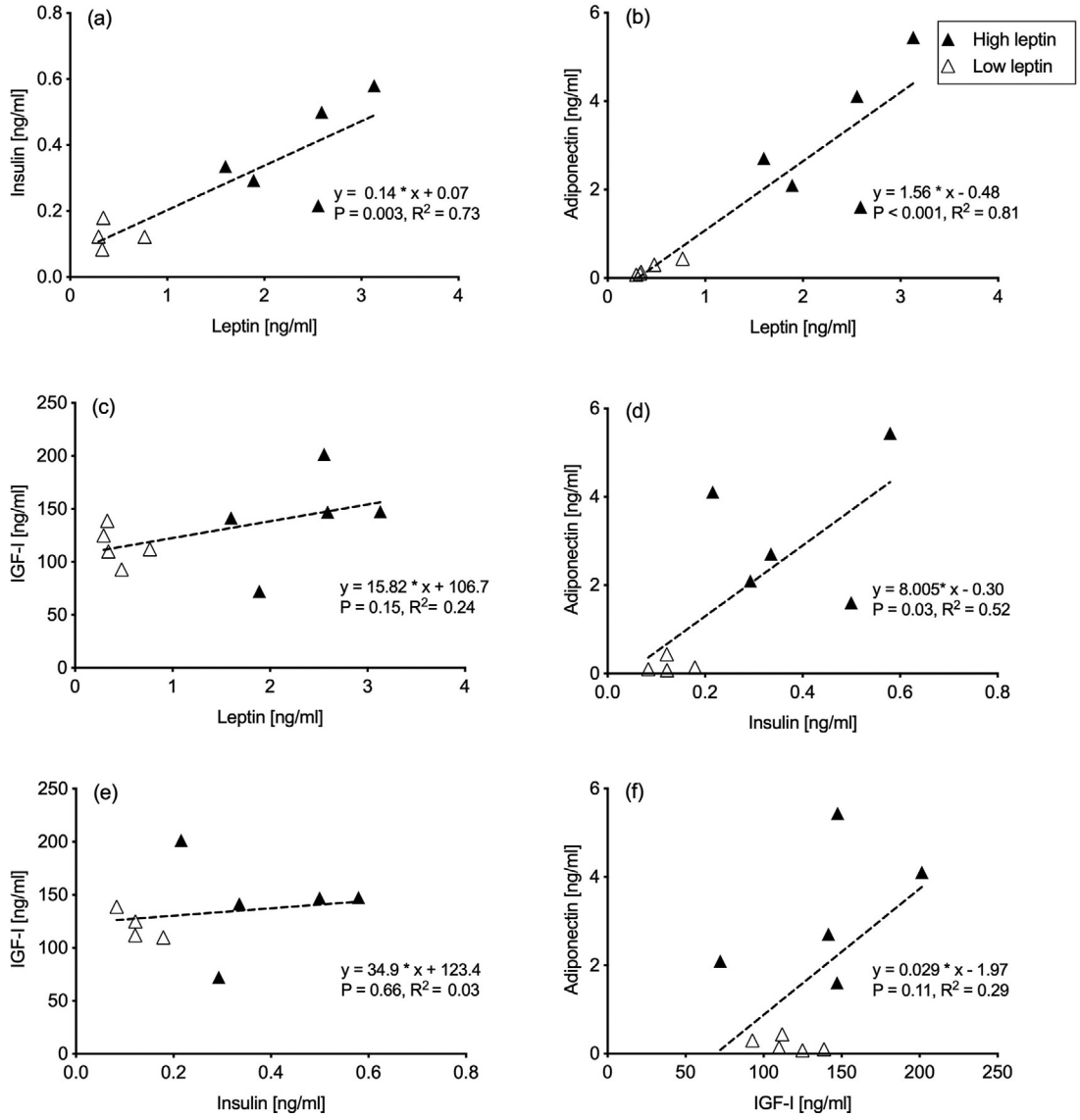

Fig. 4. Linear regression of different plasma metabolic hormone concentrations (A) – leptin and insulin, (B) – leptin and adiponectin, (C) – leptin and IGF-I, (D) – insulin and IGF-I, (E) – insulin and adiponectin, (F) – adiponectin and IGF-I. The different groups are indicated (High leptin – black triangles, Low leptin - white triangles). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

SPSS Statistics). Mann-Whitney-U test was likewise used to test for differences in AA and FA composition in BP and UF.

The effect of the sampling day and the leptin group on leptin, insulin, adiponectin and IGF-I were tested using linear mixed-effects models fit with Gaussian family distribution and identity link function in R software v.3.3.1. (LMMs, lmer function, package lme4, Bates et al., 2011). These models included the plasma concentration of the hormone as the output variable (one model for each hormone), the sampling day (continuous factor; 1–7), the leptin group (categorical factor; low or high) and their interaction as fixed factors, as well as the identity of the animals as a random factor to control for repeated measurements on the same individuals. The inclusion of non-significant interaction terms in models makes the interpretation of main effects problematic [57]. For this reason, when the interaction term between leptin group and day was not significant, it was removed from the model. When this interaction was significant, we carried out further LMMs to test the effect of the sample day on each leptin group separately. The resulting *P*-values were corrected for multiple testing using a Bonferroni correction (α * 2 post-hoc comparisons). We checked the residuals of the models graphically for normal distribution and homoscedasticity. To satisfy the model assumptions, we log-transformed the adiponectin concentration. For each model, we assessed the statistical significance of the factors by comparing the model with and without the factor included using likelihood-ratio tests (LRT). The LRT statistics follows a $\chi 2$-distribution with degrees of freedom equal to the difference in the number of parameters. To compare models with LRT, all models were fit with maximum likelihood estimation. The significance level was set at $\alpha = 0.05$.

A correlation analysis of AAs in BP and UF was performed and visualized in R version 3.6.3 using the packages corrplot and corrgram [58]. The significance level was set at $\alpha = 0.05$.

3. Results

3.1. Correlation of Body Condition Score (BCS) With Fat Thickness (FT) and Plasma Leptin

In the low leptin group, BCS and FT ranged from 1.4 to 2.3 (average BCS = 3.2, SD = ±0.6) and from 2.8 to 4.0 (average FT = 1.8, SD = ±0.3), respectively. In the high leptin group, BCS and FT

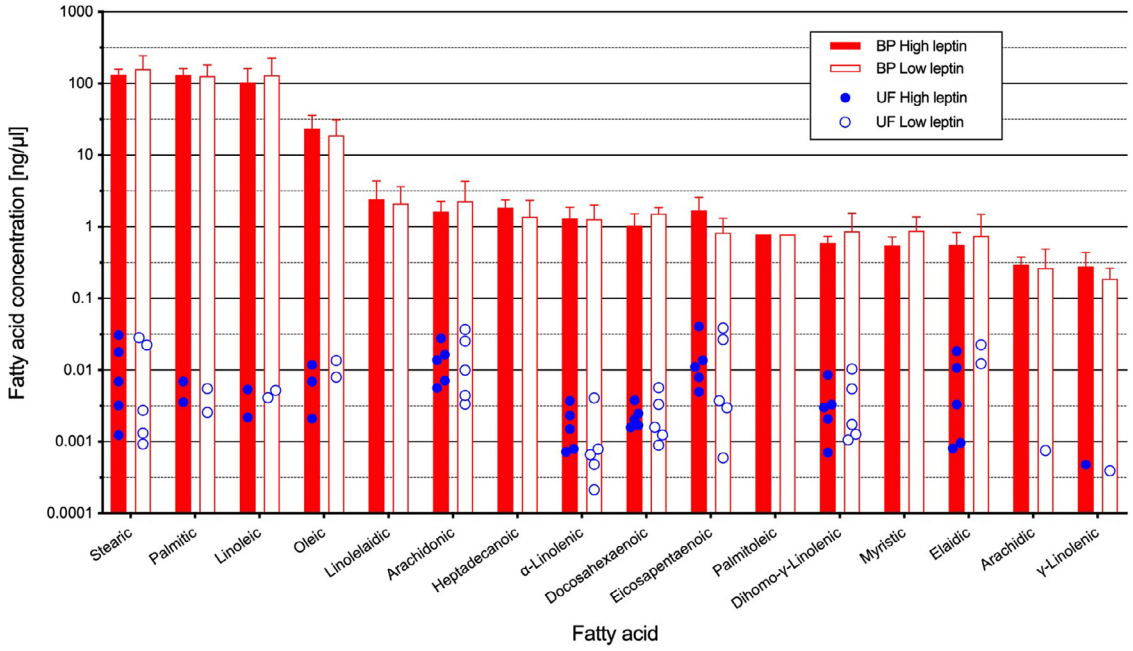

Fig. 5. Fatty acid (FA) concentrations in blood plasma (BP) and uterine fluid (UF). In BP, all FAs were detected in each animal and the respective concentrations are displayed as mean concentration ± SD for each group (High leptin – filled red bars, Low leptin- empty red bars). In the UF, several FAs were below the limit of detection and concentrations are therefore displayed as single data points (High leptin – filled blue dots, Low leptin – empty blue dots). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

ranged from 2.3 to 3.8 (average BCS = 3.3, SD = ±0.1) and from 1.4 to 1.7 (average FT = 1.6, SD = ±0.1), respectively. No significant relationship was observed between BCS and FT (linear regression: F = 0.001, P = 0.973, R^2 = 0) (Fig. 2A). In line, a linear regression model for BCS and leptin explained only 0.02% of the observed variation (F = 0.148, P = 0.710) (Fig. 2B). BCS was neither correlated to insulin, nor to adiponectin, nor to IGF-1 (data not shown).

3.2. Correlation of Metabolic Hormones

Plasma leptin concentrations did not vary according to the sampling days (LMM: χ^2_1 = 1.475, P= 0.225) (Fig. 3A). Plasma insulin and adiponectin concentrations during seven consecutive days prior to uterine flushing (Fig. 3B, C) did not differ between days (LMM: insulin, χ^2_1 = 3.269, P= 0.071; adiponectin, χ^2_1 = 0.804, P = 0.370) but the high leptin group displayed higher concentrations of insulin and adiponectin than the low leptin group (LMM: insulin, χ^2_1 = 8.445, P = .004; adiponectin, χ^2_1 = 20.197, P < 0.001). Plasma IGF-I concentrations (Fig. 3D) were affected by the interaction between day and group LMM: χ^2_1 = 4.145, P = 0.042). Further post-hoc comparisons showed that IGF-I plasma concentration increased in both leptin groups throughout the 7-day sampling period (LMM: leptin high, χ^2_1 = 23.360, P < 0.001; leptin low, χ^2_1 = 9.565, P = 0.004). This shows that IGF-I concentrations fluctuated throughout the sampling period.

There was a strong positive linear relationship between leptin and insulin (F = 19.27, P = 0.003, R^2 = 0.73) (Fig. 4A) and between leptin and adiponectin (F = 35.04, P = 0.001, R^2 = 0.81) (Fig. 4B) BP concentrations. Plasma leptin and IGF-I (Fig. 4C) were not correlated (F = 2.48, P = 0.15, R^2 = 0.24). A moderate linear relationship was observed between BP insulin and adiponectin (F = 7.61, P = 0.028, R^2= 0.53) (Fig. 4D), whereas BP insulin and IGF-I (Fig. 4E) were not correlated (F = 0.21, P = 0.656, R^2 = 0.03). There was no significant relationship between adiponectin and IGF-I in BP (F = 3.24, P = 0.11, R^2 = 0.29) (Fig. 4F).

3.3. Fatty Acids

In Fig. 5, the absolute FA concentrations in BP and UF are shown. In BP, the different FAs were detected in each animal and the respective concentrations are displayed as average FA concentrations with standard deviation for the two groups. In UF of several animals, several FAs were below the limit of detection. Therefore, the absolute concentrations of individual animals for each group are displayed as individual data points. The FA composition of neither BP nor UF differed between groups (Fig. 5). There was no significant correlation between the FA content of BP and UF except for arachidic and γ-linoleic acid where only single measurements are available. In BP, stearic (34 % of total FAs), palmitic (30 % of total FAs) and linoleic acid (27 % of total FAs) accounted for 90 % of the total FA content, followed by the far less abundant oleic acid (5 % of total FAs). Next, linolelaidic, arachidonic, heptadecanoic-, α-linolenic, docosahexaenoic, and eicosapentaenoic acid were abundant in a similar range (0.5 %, 0.5 %, 0.4 %, 0.3 %, and 0.3 % of total FAs, respectively). The three most abundant FAs in UF, which were detected and quantified in all animals, were eicosapentaenoic (0.016 ± 0.018 ng/mL), arachidonic (0.016 ± 0.013 ng/mL) and stearic acid (0.013 ± 0.014 ng/mL).

3.4. Amino Acids

Except for Ile in UF (P = .047), there was no significant difference in AA composition of BP and UF between the two leptin groups (Fig. 6A) Figure 6. In BP, the three most abundant AAs were Gly (573.0 ± 31.0 µmol), Gln (412.0 ± 18.0 µmol) and Ser (304.0 ± 17.0 µmol). Gly, Gln, and Ser accounted for 20%, 15%, and 11%, respectively, of total AAs. In UF, Glu was by far the most prominent AA (56.5 ± 6 nmol/mg TP), making up 26 % of total AAs (Fig. 6B) Figure 6. Gly with a concentration of 37.6 ± 4.0 nmol/mg TP accounted for 17% of total AAs, followed by Ala (26.6 ± 2.5 nmol/mg TP), Ser (18.4 ± 2.7 nmol/mg TP), Gln (13.5 ± 1.8 nmol/mg TP) and Asp (11.5 ± 1.3 nmol/mg TP).

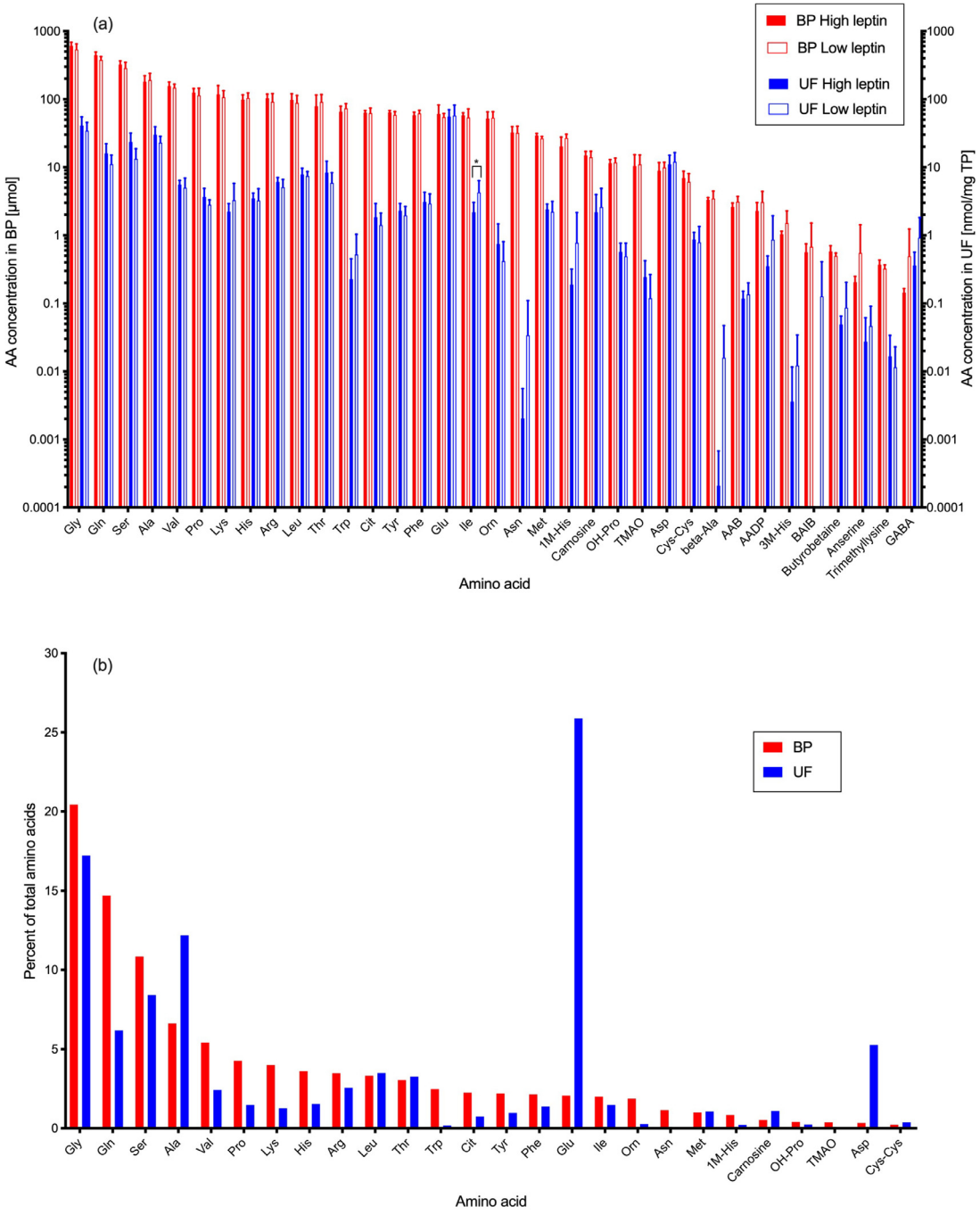

Fig. 6. Amino acids (AA) in blood plasma (BP) and uterine fluid (UF). (A) AA concentrations in the two groups in BP (High leptin – filled red bars, Low leptin – empty red bars) and UF (High leptin – filled blue bars, Low leptin – empty blue bars) are displayed as mean concentration ± SD for each group. (B) Relative abundancy of AAs in percent of total AAs in blood plasma BP (red bars) and UF (blue bars). Note that a log scale is used in graph a). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

In Fig. 7, the correlations between the different AAs are visualized. More correlations between different AAs were detected in UF than in BP. Only few significant correlations were observed between BP and UF.

4. Discussion

In the present study, the BP leptin concentrations were used as a measure of the metabolic status in mares. Interestingly and in contrast to other studies [13], in our study, neither BCS nor FT significantly positively correlated with plasma leptin concentrations. This might be attributed to the fact that the BCS is not suitable to differentiate accurately between lean body mass and fat mass [59].

General relationships between morphologic characteristics and metabolic and reproductive functions confirmed in larger cohorts of animals [10,13,60] might not always hold true for the individual. In fact, a considerable individual variance in leptin levels was documented in mares with a healthy BCS [60]. In a feeding experiment, 16 pony mares were either food restricted or supplemented in order to lose or gain weight [13]. The achieved changes in BCS over a course of 14 weeks, however, did not result in significant changes in plasma leptin concentrations. A correlation between body weight and plasma leptin concentrations could neither

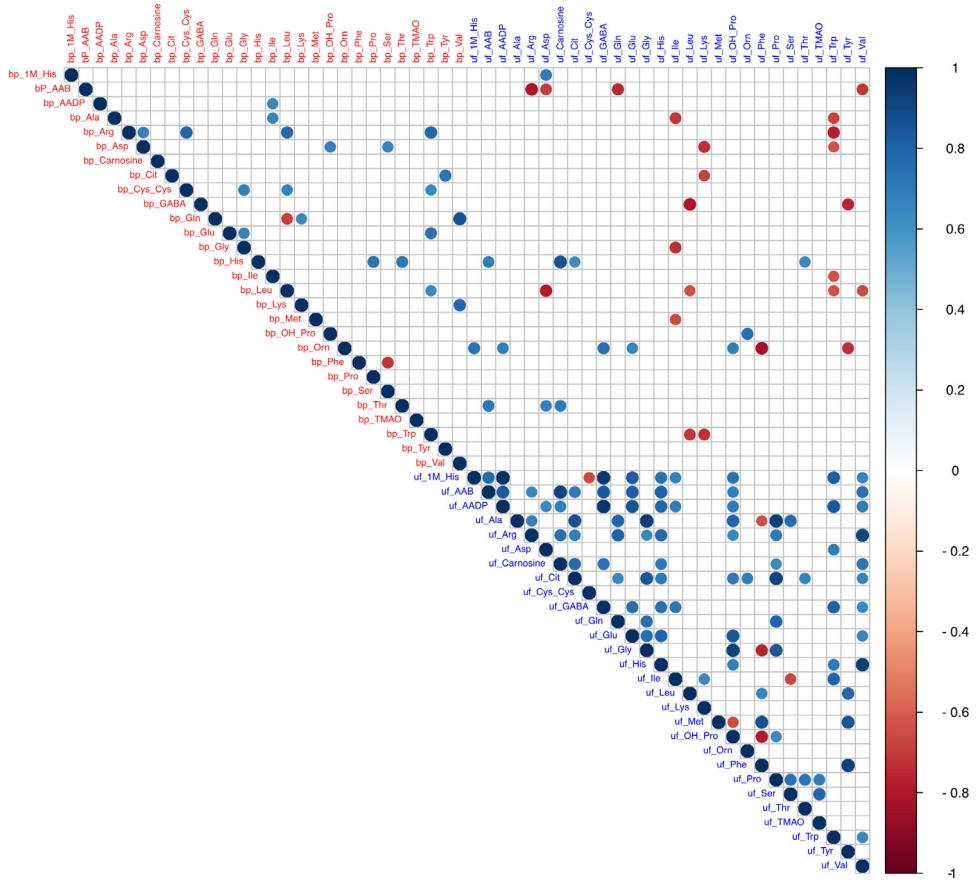

Fig. 7. Correlation matrix of blood plasma (BP) and uterine fluid (UF) amino acids (AA). The dots indicate significant positive (in blue) and negative (in red) correlations ($P \leq 0.05$). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

be confirmed in a later study with a smaller experimental group of 6 mares [8]. The above findings suggest that there might be an inherent genetic component influencing relative leptin concentrations, especially in body conditions that are within the physiological range, which was the case in our study.

Other factors such as age and season also affect plasma leptin levels and, in some cases, different effectors might offset each other. Indeed, lower mean leptin concentrations have been shown to correlate with the onset of seasonal ovarian cyclicity in 3- to 4-year-old Lipizzan fillies with stable body condition over the year [67]. This is in line with another study where mares with a constant energy balance and body weight had greater mean plasma leptin concentrations in summer compared to winter [23]. The correlation between age and circulating leptin levels in the horse is neither congruent. Whereas one study demonstrated, using an in vitro approach, that corticoid-induced release of leptin by adipocytes decreased with age [6], a trend for increased plasma leptin concentrations with age was observed in another [13]. In the present study, the oldest mare, aged 22, was in the low leptin group although she had the greatest FT of all animals.

Seasonal individual weight variation of the horses at the Chamberet breeding station has been documented with lower body weights during winter compared to summer [11]. Obesity as such is defined as a stable overweight condition throughout the year [61,62]. The BCS of the mares used in the present study was >2 and ≤ 4, which is considered within the healthy physiological range [50]. The documented differences in plasma leptin concentrations lacking a correlation to BCS might therefore be explained by individual variability. To further specify the multifactorial relationship between morphologic attributes such as FT, BCS, and serum leptin concentrations, other influencing factors for example, season, age, individual differences and reproductive status, need to be controlled for in a larger group of experimental animals.

No significant correlation between BP leptin and the AA composition of the UF was observed, nor between BP leptin and the FA composition of the UF. These findings allow for several interpretations. First, BP leptin, specifically minor changes within a physiological range, might not affect the composition of UF. Second, systemic leptin concentrations do not necessarily reflect the local presence and activity of leptin and might therefore not be a good indicator for potential hormonal effects on the uterine milieu. Third, the AA and the FA composition of the UF are very critical for embryo development and therefore need to be tightly controlled. This is in line with the finding that there is not a one-to-one relationship in the concentration of neither FAs nor AAs in BP and UF. Therefore, the UF is not a mere transudate but rather controlled for independently of systemic BP concentrations by local activation and inhibition of transporters.

A possible draw-back of or our study is the difference in the inserted flushing volumes, which required a correction of the FAs and AAs in UF according to the inserted volume and TP, respectively. The used flushing volume was relatively large to ensure flushing of the whole intrauterine surface which on the other hand implies a greater dilution of AAs and FAs concentrations. The thereby resulting relatively large dilution of the UF accounted for the difficulty to determine every FA in all mares. Uterine flushings with a volume of 70 mL have successfully been performed [63]. Hereby, the mean volume recovered was 33.6 $\pm$ 0.3 mL with a range of 7–60 mL, corresponding to an average recovery rate of 56%. Nevertheless, the average fluid recovery rate in the present study was with 83% higher than the one achieved with a volume of 70 mL. For future studies of the FAs and AAs concentrations

in UF, the relationship between inserted flushing volume, recovery rate and elution of the whole intrauterine volume needs to be optimized and standardized. Also, other options to recover undiluted fluid such as the insertion of an intrauterine tampon [64,65] should be further explored.

The finding that the FA and AA composition differed between BP and UF does not exclude the possibility of a local regulation of leptin. In contrast to BP, where linoleic, palmitic and stearic acid were most abundant, in UF, the most prominent FAs were eicosapentaenoic acid, arachidonic acid, and stearic acid. The first two FAs are precursors of the prostaglandin series 3 and 2 which are involved in anti- and proinflammatory responses, respectively. One could assume that this finding might be attributed to rapid tissue response caused by the manipulation during uterine flushing. Yet, the procedure was carried out carefully within a short period of time to minimize endometrial irritation. Furthermore, the FA profile in UF collected *ex vivo* from nonpregnant slaughtered mares [54] is congruent to the present findings. For these reasons it is rather likely that these results represent the physiological conditions. Indeed, more recent research has demonstrated an immunological function of leptin and adiponectin [66–68]. It was demonstrated that in humans, leptin and adiponectin stimulate placental secretion of cytokines and prostaglandins [35]. Prostaglandin secretion is also enhanced by leptin in rabbit oviductal cells *in vitro* [18]. A similar mechanism could hold true for the endometrium, suggesting that metabolic hormones contribute to the regulation of the uterine milieu.

Similar to the FA concentrations, the relative abundance of AAs in BP and UF differed. It is long known that AAs provided by the histotroph are essential for embryo development [69] and the availability of AAs in UF seems to be directly mirrored in the AA composition of the blastocyst [70]. In the rabbit for example, the most abundant AAs in UF are Gly, Ala, and Glu. In the rabbit day 6 blastocyst, these AAs are likewise highly represented [70]. In the present study, AAs such as Glu, a precursor of Gln, and Ala were far more abundant in UF than in BP. Glu has also been reported to be highly abundant in the UF of bovine [32,71,72], mouse [73], rabbit [70] and human [74], although less prominent than observed in this study. In pig embryos, Gln addition to the culture medium significantly improved *in vitro* development to the blastocyst stage as well as embryonic cell numbers [75]. In line with these results, higher blastocyst rates and lower expression of proapoptotic factors in pig embryos cultured with leptin and non-essential AAs such as Gln are found [76]. In bovine embryos, a preferential uptake of Glu was observed *in vitro* [77]. Bovine embryos also preferentially depleted the culture medium of Ser, Asp, Gly, Ala, and Glu [77]. Interestingly, Asp was also more abundant in the equine UF than in BP. A direct stimulatory effect of leptin on AA transporters has been documented in the intestine [39] and in the human placenta [23]. The same mechanism could therefore also play part in the AA transport of the endometrium into the histotroph and the subsequent AA uptake of the preimplantation embryo.

Several studies have revealed linear correlations between certain AAs. For example, the BP concentrations of the branched chained AAs Leu and Ile or Val are closely linked in rats [78] and humans [79]. This is in line with the fact that AA transporters react with whole groups of AAs, for example, small, large, cationic, anionic, or neutral AAs. Indeed, in our study, we could find that the neutral AA Ala in UF was positively correlated with other neutral AAs, namely Gly, Gln, Pro and Ser.

From our results, we cannot exclude a local effect of metabolic hormones, especially leptin, on UF composition. Since these impacts cannot be deduced from BP hormone concentrations, further investigations need to focus on local hormone action including gene expression analyses of the endometrium.

Acknowledgments

We greatly acknowledge the expertise of Elodie Mandel-Briefer and Meret Schmidhauser for assistance in statistical analysis. We likewise express our gratitude to Ralph Schlapbach, Stefan Schauer and Endre Laczko from the Functional Genomic Center Zurich (FGCZ) for the GC-MS analyses.

Financial Disclosure

This work was partially funded by “Stiftung für das Pferd” (PR2016/01). A Swiss Government Excellence Scholarship was granted to VM.

References

[1] Fontana R, Della Torre S. The deep correlation between energy metabolism and reproduction: a view on the effects of nutrition for women fertility. Nutrients 2016;8:1–34.
[2] Marie M, Findlay PA, Thomas L, Adam CL. Daily patterns of plasma leptin in sheep: effects of photoperiod and food intake. J Endocrinol 2001;170:277–86.
[3] Cammisotto PG, Bukowiecki LJ. Mechanisms of leptin secretion from white adipocytes. Am J Physiol-Cell Ph 2002;283:C244–CC50.
[4] Cammisotto PG, Gelinas Y, Deshaies Y, Bukowiecki LJ. Regulation of leptin secretion from white adipocytes by free fatty acids. Am J Physiol-Endoc M 2003;285:E521–E5E6.
[5] Kanu A, Fain JN, Bahouth SW, Cowan GS Jr. Regulation of leptin release by insulin, glucocorticoids, G(i)-coupled receptor agonists, and pertussis toxin in adipocytes and adipose tissue explants from obese humans in primary culture. Metabolism 2003;52:60–6.
[6] Kędzierski W, Łopuszyński W, Wydrych J. Age- and glucocorticoid-dependent leptin release by horse adipose tissue: in vitro study. J Equine Vet Sci 2017;56:104–9.
[7] Cowley MA, Smart JL, Rubinstein M, Cordan MG, Diano S, Horvath TL, et al. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature 2001;411:480–4.
[8] Buff PR, Messer NT, Cogswell AM, Johnson PJ, Keisler DH, Ganjam VK. Seasonal and pulsatile dynamics of thyrotropin and leptin in mares maintained under a constant energy balance. Domest Anim Endocrinol 2007;33:430–6.
[9] Klocek-Gorka B, Szczesna M, Molik E, Zieba DA. The interactions of season, leptin and melatonin levels with thyroid hormone secretion, using an in vitro approach. Small Ruminant Res 2010;91:231–5.
[10] Salazar-Ortiz J, Camous S, Briant C, Lardic L, Chesneau D, Guillaume D. Effects of nutritional cues on the duration of the winter anovulatory phase and on associated hormone levels in adult female Welsh pony horses (Equus caballus). Reprod Biol Endocrinol 2011;9:130.
[11] Robles M, Gautier C, Mendoza L, Peugnet P, Dubois C, Dahirel M, et al. Maternal nutrition during pregnancy affects testicular and bone development, glucose metabolism and response to overnutrition in weaned horses up to two years. PLoS ONE 2017;12:e0169295.
[12] Morley SA, Murray JA. Effects of body condition score on the reproductive physiology of the broodmare: a review. J Equine Vet Sci 2014;34:842–53.
[13] Buff PR, Dodds AC, Morrison CD, Whitley NC, McFadin EL, Daniel JA, et al. Leptin in horses: tissue localization and relationship between peripheral concentrations of leptin and body condition. J Anim Sci 2002;80:2942–8.
[14] Gentry LR, Thompson D, Gentry G, Vecchio R, Davis KA, Vecchio PMD. The relationship between body condition score and ultrasonic fat measurements in mares of high versus low body condition. J Equine Vet Sci 2004;24:198–203.
[15] Kearns CF, McKeever KH, Roegner V, Brady SM, Malinowski K. Adiponectin and leptin are related to fat mass in horses. Vet J 2006;172:460–5.
[16] Wooldridge AA, Edwards HG, Plaisance EP, Applegate R, Taylor DR, Taintor J, et al. Evaluation of high-molecular weight adiponectin in horses. Am J Vet Res 2012;73:1230–40.
[17] Albrizio M, Roscino MT, Trisolini C, Binetti F, Rizzo A, Sciorsci RL. The expression of leptin receptor in the ovary of the queen: leptin receptor expression in queen ovary. Res Vet Sci 2013;95:629–31.
[18] Zerani M, Boiti C, Dall'Aglio C, Pascucci L, Maranesi M, Brecchia G, et al. Leptin receptor expression and in vitro leptin actions on prostaglandin release and nitric oxide synthase activity in the rabbit oviduct. J Endocrinol 2005;185:319–25.
[19] Alfer J, Muller-Schottle F, Classen-Linke I, von Rango U, Happel L, Beier-Hellwig K, et al. The endometrium as a novel target for leptin: differences in fertility and subfertility. Mol Hum Reprod 2000;6:595–601.
[20] Cervero A, Horcajadas JA, Martin J, Pellicer A, Simon C. The leptin system during human endometrial receptivity and preimplantation development. J Clin Endocrinol Metab 2004;89:2442–51.
[21] Moreira F, Gheller SMM, Mondadori RG, Varela AS, Corcini CD, Lucia T. Presence of leptin and its receptor in the hypothalamus, uterus and ovaries of swine females culled with distinct ovarian statuses and parities. Reprod Domest Anim 2014;49:1074–8.

[22] Balogh O, Staub LP, Gram A, Boos A, Kowalewski MP, Reichler IM. Leptin in the canine uterus and placenta: possible implications in pregnancy. Reprod Biol Endocrinol 2015;13.
[23] Farley DM, Choi J, Dudley DJ, Li C, Jenkins SL, Myatt L, et al. Placental amino acid transport and placental leptin resistance in pregnancies complicated by maternal obesity. Placenta 2010;31:718–24.
[24] Tsai P-JS, Davis J, Bryant-Greenwood G. Systemic and placental leptin and its receptors in pregnancies associated with obesity. Reproductive Sciences 2015;22:189–97.
[25] Aguilar J, Reyley M. The uterine tubal fluid: secretion, composition and biological effects. Animal Reproduction 2005;2:91–105.
[26] Bazer FW. Uterine protein secretions: relationship to development of the conceptus. J Anim Sci 1975;41:1376–82.
[27] Gao HJ, Wu GY, Spencer TE, Johnson GA, Li XL, Bazer FW. Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine lumenal flushings of cyclic and pregnant ewes. Biol Reprod 2009;80:86–93.
[28] Groebner AE, Rubio-Aliaga I, Schulke K, Reichenbach HD, Daniel H, Wolf E, et al. Increase of essential amino acids in the bovine uterine lumen during preimplantation development. Reproduction 2011;141:685–95.
[29] Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. Theriogenology 2005;64:992–1006.
[30] Iritani A, Sato E, Nishikaw Y. Secretion rates and chemical composition of oviduct and uterine fluids in sows. J Anim Sci 1974;39:582–7.
[31] Forde N, Simintiras CA, Sturmey R, Mamo S, Kelly AK, Spencer TE, et al. Amino acids in the uterine luminal fluid reflects the temporal changes in transporter expression in the endometrium and conceptus during early pregnancy in cattle. Plos One 2014;9:e100010.
[32] Hugentobler SA, Diskin MG, Leese HJ, Humpherson PG, Watson T, Sreenan JM, et al. Amino acids in oviduct and uterine fluid and blood plasma during the estrous cycle in the bovine. Mol Reprod Dev 2007;74:445–54.
[33] Hugentobler SA, Humpherson PG, HJ Leese, Sreenan JM, Morris DG. Energy substrates in bovine oviduct and uterine fluid and blood plasma during the oestrous cycle. Mol Reprod Dev 2008;75:496–503.
[34] Zhang FM, Basinski MB, Beals JM, Briggs SL, Churgay LM, Clawson DK, et al. Crystal structure of the obese protein leptin-E100. Nature 1997;387:206–9.
[35] Lappas M, Permezel M, Rice GE. Leptin and adiponectin stimulate the release of proinflammatory cytokines and prostaglandins from human placenta and maternal adipose tissue via nuclear factor-kappa B, peroxisomal proliferator-activated receptor-gamma and extracellularly regulated kinase 1/2. Endocrinology 2005;146:3334–42.
[36] Gonzalez RR, Caballero-Campo P, Jasper M, Mercader A, Devoto L, Pellicer A, et al. Leptin and leptin receptor are expressed in the human endometrium and endometrial leptin secretion is regulated by the human blastocyst. J Clin Endocrinol Metab 2000;85:4883–8.
[37] Tanaka T, Umesaki N. Leptin regulates the proliferation and apoptosis of human endometrial epithelial cells. Int J Mol Med 2008;22:683–9.
[38] Lange-Consiglio A, Arrighi S, Fiandanese N, Pocar P, Aralla M, Bosi G, et al. Follicular fluid leptin concentrations and expression of leptin and leptin receptor in the equine ovary and in vitro-matured oocyte with reference to pubertal development and breeds. Reprod Fertil Dev 2013;25:837–46.
[39] Fanjul C, Barrenetxe J, De Pablo-Maiso L, Lostao MP. In vivo regulation of intestinal absorption of amino acids by leptin. J Endocrinol 2015;224:17–23.
[40] Jansson N, Greenwood SL, Johansson BR, Powell TL, Jansson T. Leptin stimulates the activity of the system A amino acid transporter in human placental villous fragments. The J Clin Endocrinol & Metabolism 2003;88:1205–11.
[41] Madeja ZE, Warzych E, Peippo J, Lechniak D, Switonski M. Gene expression and protein distribution of leptin and its receptor in bovine oocytes and preattachment embryos produced in vitro. animal 2009;3:568–78.
[42] Gibson C, De Ruijter-Villani M, Rietveld J, Stout TAE. Amino acid transporter expression in the endometrium and conceptus membranes during early equine pregnancy. Reprod Fert Develop 2018;30:1675–88.
[43] Drabkova P, Andrlova L, Hampl R, Kandar R. Amino acid metabolism in human embryos. Physiol Res 2016;65:823–32.
[44] Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, et al. Non-invasive amino acid turnover predicts human embryo developmental capacity. Hum Reprod 2002;17:999–1005.
[45] Partridge RJ, Leese HJ. Consumption of amino acids by bovine preimplantation embryos. Reprod Fertil Dev 1996;8:945–50.
[46] Van Winkle LJ. Amino acid transport regulation and early embryo development. Biol Reprod 2001;64:1–12.
[47] Sessions-Bresnahan DR, Heuberger AL, Carnevale EM. Obesity in mares promotes uterine inflammation and alters embryo lipid fingerprints and homeostasis†. Biol Reprod 2018;99:761–72.
[48] Leese HJ, Hugentobler SA, Gray SM, Morris DG, Sturmey RG, Whitear SL, et al. Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. Reprod Fert Develop 2008;20:1–8.
[49] Betteridge KJ, Eaglesome MD, Mitchell D, Flood PF, Beriault R. Development of horse embryos up to twenty two days after ovulation: observations on fresh specimens. J Anat 1982;135:191–209.
[50] Arnaud G. Notation de l'état corporel des chevaux de selle et de sport. Guide pratique. Paris Cedex: INRA; 2000. p. 1–40.
[51] Groebner AE, Rubio-Aliaga I, Schulke K, Reichenbach HD, Daniel H, Wolf E, et al. Increase of essential amino acids in the bovine uterine lumen during preimplantation development. Reproduction 2011;141:685–95.
[52] Robles M, Nouveau E, Gautier C, Mendoza L, Dubois C, Dahirel M, et al. Maternal obesity increases insulin resistance, low-grade inflammation and osteochondrosis lesions in foals and yearlings until 18 months of age. PLoS ONE 2018;13:e0190309.
[53] Piechotta M, Mysegades W, Ligges U, Lilienthal J, Hoeflich A, Miyamoto A, et al. Antepartal insulin-like growth factor 1 and insulin-like growth factor binding protein 2 concentrations are indicative of ketosis in dairy cows. J Dairy Sci 2015;98:3100–9.
[54] Drews B, Milojevic V, Giller K, Ulbrich SE. Fatty acid profile of blood plasma and oviduct and uterine fluid during early and late luteal phase in the horse. Theriogenology 2018;114:258–65.
[55] Harder U, Koletzko B, Peissner W. Quantification of 22 plasma amino acids combining derivatization and ion-pair LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 2011;879:495–504.
[56] Gucciardi A, Pirillo P, Di Gangi IM, Naturale M, Giordano G. A rapid UPLC-MS/MS method for simultaneous separation of 48 acylcarnitines in dried blood spots and plasma useful as a second-tier test for expanded newborn screening. Anal Bioanal Chem 2012;404:741–51.
[57] Engqvist L. Commentary. Anim Behav. 2005;70:967–71.
[58] Friendly M. Corrgrams. The American Statistician 2002;56:316–24.
[59] Shah NR, Braverman ER. Measuring adiposity in patients: the utility of body mass index (BMI), percent body fat, and leptin. PLoS ONE 2012;7:e33308.
[60] Gentry LR, Thompson DL Jr, Gentry GT Jr, Davis KA, Godke RA, Cartmill JA. The relationship between body condition, leptin, and reproductive and hormonal characteristics of mares during the seasonal anovulatory period1. J Anim Sci 2002;80:2695–703.
[61] Giles SL, Rands SA, Nicol CJ, Harris PA. Obesity prevalence and associated risk factors in outdoor living domestic horses and ponies. Peerj 2014;2.
[62] Jensen RB, Danielsen SH, Tauson AH. Body condition score, morphometric measurements and estimation of body weight in mature Icelandic horses in Denmark. Acta Vet Scand 2016;58.
[63] LeBlanc MM, Magsig J, Stromberg AJ. Use of a low-volume uterine flush for diagnosing endometritis in chronically infertile mares. Theriogenology 2007;68:403–12.
[64] Smits K, Willems S, Van Steendam K, Van De Velde M, De Lange V, Ververs C, et al. Proteins involved in embryo-maternal interaction around the signalling of maternal recognition of pregnancy in the horse. Scientific reports 2018;8:5249.
[65] Troedsson MH, Liu IK. Measurement of total volume and protein concentration of intrauterine secretion after intrauterine inoculation of bacteria in mares that were either resistant or susceptible to chronic uterine infection. Am J Vet Res 1992;53:1641–4.
[66] Carbone F, La Rocca C, Matarese G. Immunological functions of leptin and adiponectin. Biochimie 2012;94:2082–8.
[67] Otero M, Lago R, Gomez R, Dieguez C, Lago F, Gomez-Reino J, et al. Towards a pro-inflammatory and immunomodulatory emerging role of leptin. Rheumatology 2006;45:944–50.
[68] Stofkova A. Leptin and adiponectin: from energy and metabolic dysbalance to inflammation and autoimmunity. Endocr Regul 2009;43:157–68.
[69] Bavister BD, Leibfried ML, Lieberman G. Development of pre-implantation embryos of the golden-hamster in a defined culture-medium. Biol Reprod 1983;28:235–47.
[70] Miller JGO, Schultz GA. Amino-acid content of preimplantation rabbit embryos and fluids of the reproductive-tract. Biol Reprod 1987;36:125–9.
[71] França MR, da Silva MIS, Pugliesi G, Van Hoeck V, Binelli M. Evidence of endometrial amino acid metabolism and transport modulation by peri-ovulatory endocrine profiles driving uterine receptivity. J Anim Sci Biotechno 2017:54.
[72] Meier S, Mitchell MD, Walker CG, Roche JR, Verkerk GA. Amino acid concentrations in uterine fluid during early pregnancy differ in fertile and subfertile dairy cow strains. J Dairy Sci 2014;97:1364–76.
[73] Eckert JJ, Porter R, Watkins AJ, Burt E, Brooks S, Leese HJ, et al. Metabolic induction and early responses of mouse blastocyst developmental programming following maternal low protein diet affecting life-long health. PLoS ONE 2012;7:e52791.
[74] Kermack AJ, Finn-Sell S, Cheong YC, Brook N, Eckert JJ, Macklon NS, et al. Amino acid composition of human uterine fluid: association with age, lifestyle and gynaecological pathology. Hum Reprod 2015;30:917–24.
[75] Chen PR, Redel BK, Spate LD, Ji TM, Salazar SR, Prather RS. Glutamine supplementation enhances development of in vitro-produced porcine embryos and increases leucine consumption from the medium. Biol Reprod 2018;99:938–48.
[76] Li XX, Lee DS, Kim KJ, Lee JH, Kim EY, Park JY, et al. Leptin and nonessential amino acids enhance porcine preimplantation embryo development in vitro by intracytoplasmic sperm injection. Theriogenology 2013;79:291–8.
[77] Lee ES, Fukui Y. Synergistic effect of alanine and glycine on bovine embryos cultured in a chemically defined medium and amino acid uptake by vitro-produced bovine morulae and blastocysts. Biol Reprod 1996;55:1383–9.
[78] Noguchi Y, Zhang Q-W, Sugimoto T, Furuhata Y, Sakai R, Mori M, et al. Network analysis of plasma and tissue amino acids and the generation of an amino index for potential diagnostic use. The Am J Clin Nutrition 2006;83 513S-519S.
[79] Armstrong MD, Stave U. A study of plasma free amino acid levels. V. Correlations among the amino acids and between amino acids and some other blood constituents. Metabolism 1973;22:827–33.