

Involvement of somatotrophic hormones in the postpartum regulation of ovarian activity in mares

Maria Melchert^{a,*}, Jörg Aurich^a, Reinhard Ertl^b, Ursula Reichart^b, Ingrid Walter^{b,c}, Camille Gautier^a, Martim Kaps^a, Christine Aurich^a

^a Center for Animal Reproduction, Vetmeduni Vienna, Veterinärplatz 1, 1210 Vienna, Austria

^b Vetcore Facility for Research, Vetmeduni Vienna, Veterinärplatz 1, 1210 Vienna, Austria

^c Center for Pathobiology, Institute of Morphology, Vetmeduni Vienna, Veterinärplatz 1, 1210 Vienna, Austria

ARTICLE INFO

Key words:

Horse
Postpartum ovulation
Placenta
Follicular growth
IGF-1

ABSTRACT

Mares resume ovarian activity rapidly after foaling. Besides follicle-stimulating hormone (FSH) and luteinizing hormone (LH), the pituitary synthesizes prolactin and growth hormone which stimulate insulin-like growth factor (IGF) synthesis in the liver. We tested the hypothesis that follicular growth is initiated already antepartum, mares with early and delayed ovulation differ in IGF-1 release and that there is an additional IGF-1 synthesis in the placenta. Plasma concentrations of LH, FSH, IGF-1, IGF-2, activin and prolactin. IGF-1, IGF-2, prolactin and their receptors in placental tissues were analyzed at the mRNA and protein level. Follicular growth was determined from 15 days before to 15 days after foaling in 14 pregnancies. Mares ovulating within 15 days postpartum formed group OV (n=5) and mares not ovulating within 15 days group NOV (n=9). Before foaling, follicles with a diameter >1 cm were present in all mares and their number increased over time (p<0.05). Follicle growth after foaling was more pronounced in OV mares (day p<0.001, group p<0.05, day x group p<0.05) in parallel to an increase in LH concentration (p<0.001, day x group p<0.001) while FSH increased (p<0.001) similarly in both groups. Plasma concentrations of IGF-1 and prolactin peaked one day after foaling (p<0.001). The IGF-1 mRNA abundance was higher in the allantochorion but lower in the amnion of OV versus NOV mares (group p=0.01, localization x group p<0.01). The IGF-1 receptor mRNA was most abundant in the allantochorion (p<0.001) and IGF-1 protein was expressed in placental tissue without differences between groups. In conclusion, follicular growth in mares is initiated before foaling and placental IGF-1 may enhance resumption of ovulatory cycles.

1. Introduction

Horse mares resume cyclic ovarian activity rapidly after foaling and much faster than other domestic animal species. Despite nursing a foal, more than 80% of mares ovulate within 14 days after foaling and continue to cycle thereafter [e.g., 1–4]. The anterior pituitary plays a key role in stimulating ovarian function and besides follicle-stimulating hormone (FSH) and luteinizing hormone (LH) also synthesizes and releases growth hormone (GH). This in turn stimulates hepatic production and secretion of insulin-like growth factor-1 (IGF-1). In early pregnancy, IGF-1 is important for fetal growth and detectable in the allantochorion [5,6]. With progressing pregnancy, however, the expression of IGF-1 in the allantochorion decreases [7].

In mares, the intrafollicular IGF-1 concentration is positively

correlated with follicle size [8,9]. Together with intrafollicular estradiol, activin-A and inhibin-A, IGF-1 is involved in the regulation of follicular deviation [10,11]. The IGF-1 concentration in plasma increases in the last weeks before foaling [12] and together with an abrupt peripartum release of prolactin [12–15] has been suggested to stimulate follicular growth after foaling. In contrast, prolactin alone appears to be without direct effects on follicular growth in postpartum mares [16]. Its main function in the peripartum period in horses is the induction of galactogenesis [17].

In ruminants, key adaptations of early lactation include an increase in the release of GH and a decrease in plasma IGF-1 concentrations. These endocrine changes are beneficial for energy metabolism but suppress reproductive functions [18,19]. Growth hormone, besides its function on growth and development, stimulates ovarian activity. Parts

* Corresponding author at: Center for Animal Reproduction, Vetmeduni Vienna, Veterinärplatz 1, 1210 Vienna, Austria.

E-mail address: maria.melchert@vetmeduni.ac.at (M. Melchert).

<https://doi.org/10.1016/j.domaniend.2024.106852>

Received 18 March 2024; Received in revised form 23 April 2024; Accepted 25 April 2024

Available online 26 April 2024

0739-7240/© 2024 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

of these actions are mediated via IGF-1 released from the liver in response to GH [20,21]. Directly or via IGF-1, GH stimulates ovarian steroidogenesis, either independent from gonadotrophins or by potentiating the stimulatory effects of gonadotrophins [22]. In cattle, the number of growing follicles is closely associated with plasma IGF-1 concentrations [21,23].

In pigs, the release of GH and prolactin also increases after parturition [24,25], but different from dairy cows, sows can meet the energy demands of lactation with adequate feed intake [26]. In this species, an increased IGF-1 concentration postpartum may be a consequence of elevated GH release. Concentration of IGF-1 in plasma then decreases throughout lactation [27]. The IGF-1 plasma concentration correlates with its concentration in follicular fluid and the degree of oocyte maturation [28,29]. Feed restriction or a marked depletion of body energy stores during lactation result in decreased IGF-1 concentration at weaning and a subsequent reduction in follicle growth and oocyte quality [29,30].

This study followed the hypothesis that in mares, follicular growth is initiated already before foaling, and mares with early and delayed onset of postpartum ovarian cyclicity differ in somatotrophic hormone synthesis and release. We therefore determined the concentrations of prolactin and somatotrophic hormones in blood and their abundance in placental tissue of peripartum mares. Ovarian follicular growth was assessed before and after foaling.

2. Materials and methods

2.1. Animals

The study included seven pregnant Haflinger mares over two consecutive pregnancies; thus 14 complete data sets were analyzed. At the beginning of the study, mares were between 4 and 15 years old (9.6 ± 3.7 years) with a body weight between 505 and 565 kg (540 ± 18 kg) before foaling and 451 and 504 kg (473 ± 13 kg) after foaling. Bodyweight was determined once weekly. All mares had been bred by natural cover to different stallions selected by their owner. During pregnancy, they were housed in individual boxes with daily groupwise access to an outdoor paddock on a private Haflinger stud. On day 300 of pregnancy, mares were transported to Vetmeduni Vienna (16 km) where they were kept similar as in their home stable and were allowed 10 days without experimental procedures for habituation. Mares were kept under ambient light, i.e., no light program was applied. They were fed hay and mineral supplements to their individual requirements. Water was freely available at all times.

2.2. Experimental design

The study was approved by the Austrian Federal Ministry for Education, Science and Research (license number BMBWF-68.205/00211-V/3b/2018).

From day 310 of pregnancy onwards, transrectal ultrasonographic examinations of the ovaries and uterus were performed at five-day intervals. The number and size of detectable follicles on both ovaries was determined and the combined thickness of uterus and placenta (CTUP) was measured following routine procedures. Follicle size was defined as the mean follicle diameter in two perpendicular dimensions [31]. When an increase in follicular size occurred between two ultrasound examinations, examinations were continued at daily intervals. After foaling, follicle numbers and size were determined daily until either the first postpartum ovulation or until day 15 postpartum, whatever occurred first. Blood samples were collected with each ultrasound examination. Based on the date of the first postpartum ovulation date, mares were assigned to two groups: mares which ovulated within 15 days after foaling formed group OV ($n=5$) and mares not ovulating within 15 days after foaling were assigned to group NOV ($n=9$).

All foalings were observed and uneventful without obstetrical

intervention. Time from rupture of the amnion to complete delivery of the foal (stage 2 of labor) ranged from 5 to 19 min (9.1 ± 3.9 min) and did not differ between groups. After foaling, mares were dewormed (200 µg Ivermectin and 1.5 mg Praziquantel; Equimax, Virbac, Carros, France) and the foals were checked for general health and for signs of prematurity. Foals received tetanus antitoxin s.c. (Equilis Tetanus-Serum; 3.000 IU, MSD, Unterschleissheim, Germany) and at 18 hours of life, the IgG concentration in foals' plasma was measured by Densimeter (Model 590a, Animal Reproduction Systems, Ontario, CA, USA). Foals did not show any signs of prematurity and IgG concentration in plasma was adequate in all foals (1957 ± 115 mg/dL). All mares and foals were healthy throughout the study period. As soon as the placenta was expelled, it was weighed and measured, and placental tissue samples were collected (see below). An endometrial swab was collected immediately after shedding of the placenta and submitted to bacteriological culture to exclude placentitis. No pathogenic agents were detected in any of the mares.

2.3. Blood sampling and endocrine analyses

Blood was collected from the jugular vein into lithium heparin and serum vacutainer tubes (Vacuette, Greiner, Kremsmünster, Austria), centrifuged immediately after collection at $3000 \times g$ for 10 min at 4°C and stored at -20°C until analysis. Serum FSH and LH concentrations were analysed by radioimmunoassays at Endolytics (Fort Collins, CO, USA), previously validated for horses [13,32]. The intra-assay coefficients of variation were 3.2% and 6.7%, and the interassay coefficients of variation were 11.0% and 18.8% for LH and FSH, respectively. The sensitivity of the assay was 0.98 ng/mL for LH and 2.3 ng/mL for FSH. Plasma activin A concentration was measured by a sandwich ELISA validated for equine plasma [33] (DAC00B, R&D Systems, Minneapolis, MN, USA). Concentrations of activin A were expressed in terms of recombinant human activin A. The sensitivity of the assay was 5.0 pg/mL and the intra-assay and interassay coefficients of variations were 13.2 and 27.5%, respectively. The concentration of IGF-1 in plasma was measured with an insulin-like growth factor binding proteins (IGFBP) blocking ELISA (IGF-1 ELISA DEE 020, Demeditec Diagnostics, Kiel, Germany). After neutralization of the sample, the IGF-2 present in high excess occupies the IG binding sites of the binding proteins. This makes the now free IGF-1 detectable. The sensitivity of the assay was 0.64 ng/mL and the intra- and interassay coefficients of variations were 4.0 and 2.9%, respectively. Similarly, IGF-2 concentration was measured with an ELISA (IGF-2 ELISA DEE 030, Demeditec Diagnostics, Kiel, Germany) using two specific and highly affine antibodies. First, the IGFBPs were diluted and then blocked by the IGF-1 excess. Because of the low cross-reactivity of the IGF-2 antibody with IGF-1, the high excess of IGF-1 does not interfere with the specific interaction with IGF-2. The sensitivity of the assay was 0.3 ng/mL and the intra- and interassay coefficients of variations were 2.4 and 2.2%, respectively. Prolactin concentration in plasma was measured with an ELISA (Prolactin ELISA DE 1291, Demeditec Diagnostics, Kiel, Germany) validated for horse plasma in the authors' laboratory. The sensitivity of the assay was 0.17 ng/mL and the intra- and interassay coefficients of variations were 11.0 and 14.6%, respectively.

2.4. Placenta sampling and analyzing

Directly after expulsion, the placental weight was determined, and the surface area measured as described previously [34]. The chorioallantois was checked for abnormalities in color, thickened areas, presence of exudate and areas devoid of villi. Eight full thickness samples (3×3 cm) were collected from the amnion and five from the chorioallantois (cervical star area, cranial uterine body, base and tip of both uterine horns, umbilical cord) as described previously [35], fixed in 4% neutral buffered formaldehyde for 48 hours and thereafter stored in 70% ethanol before tissue embedding in paraffin. Samples for real-time

quantitative polymerase chain reaction (qPCR) were collected from the same locations, immersed in liquid nitrogen and transferred to a -80°C freezer for further storage.

2.5. Real-time qPCR

Tissue samples were cut into pieces of approximately 3 × 3 × 3 mm size and placed into 2-mL screw cap tubes pre-filled with 1.4 mm ceramic beads (Qiagen, Hilden, Germany) and 600 µL TRI Reagent (Zymo Research, Irvine, CA, USA). The samples were homogenized with a MagNA Lyser instrument (Roche, Rotkreuz, Switzerland) at 6500 rpm for 30 sec. RNA extraction and DNase treatment were conducted with the Direct-zol RNA Miniprep Kit (Zymo Research) according to the manufacturer's protocol. The RNA concentrations were measured on a DS-11 FX+ spectrophotometer (DeNovix, Wilmington, DE, USA) and RNA integrity was assessed on a 4200 TapeStation with the RNA ScreenTape assay or the High Sensitivity RNA ScreenTape assay (Agilent, Santa Clara, CA, USA) for lowly concentrated samples. Reverse transcription (RT) using 1 µg total RNA was performed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA, USA) following the recommended protocol. No-RT controls (without RT enzyme) were included to monitor the amplification of contaminating DNA. RT-qPCR was done in 15-µL reaction volumes including 1x HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia), 200 nM of each primer and 20 ng cDNA. Primer sequences [36] are listed in Table 1. The PCR reactions were pipetted in 384-well plates with the epMotion 5075 automated pipetting system (Eppendorf, Hamburg, Germany). The samples were analyzed in triplicates on a qTOWER3 84 real-time PCR cycler (Analytik Jena, Jena, Germany) with the following temperature profile: 95°C for 12 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a melting curve analysis step (60°C – 95°C). Four candidate reference genes (*ACTB*, *GAPDH*, *PSMB4* and *SNRPD3*) were included for normalization [36]. The RefFinder tool [37] was used to evaluate the reference gene stability, identifying *PSMB4* and *SNRPD3* as the most stably expressed genes. Target gene Cq-values were corrected for PCR reaction efficiencies and normalized to the geometric mean of *PSMB4* and *SNRPD3*. Relative expression changes were calculated with the comparative 2^{-ΔΔCT} method [38].

2.6. Immunohistochemistry

Formaldehyde fixed samples were embedded in paraffin and cut at 3 µm. Paraffin sections were deparaffinized and rehydrated. Staining of IGF-1 and prolactin receptor was done in the amnion and both uterine horns of the chorioallantois. For IGF-1 staining, a polyclonal primary antibody (GroPep, Adelaide, Australia) was used at 1:50 dilution. For immunohistochemical staining, endogenous peroxidases were inhibited with 0.6% H₂O₂ in methanol for 15 minutes at room temperature, afterwards samples were washed with tap water 10 times. Thereafter incubation in 1.5% normal goat serum (Sigma Aldrich) in PBS for 30 minutes was used to minimize non-specific antibody binding. Sections were incubated with the primary antibody (IGF-1) overnight at 4°C. The next day, sections were washed in PBS solution and treated with secondary antibody (BrightVision Poly-HRP-anti-rabbit; ImmunoLogic, Duiven, Netherlands) for 30 minutes at room temperature. Subsequently, sections were washed and developed in diaminobenzidine substrate (Quanto, Richard Allan Scientific) according to the manufacturer's information for 5 minutes at room temperature. Thereafter, sections were washed in distilled water and counterstained with Mayer's haemalaun. A negative control was incubated with PBS instead of the primary antibody to assess unspecific binding of the secondary antibody (rabbit). The antibody was previously validated for IGF-1 staining in equine embryos [39]. For immunohistochemical staining of the prolactin receptor (PLR), monoclonal mouse-anti-prolactin receptor (Clone U5, Invitrogen) was used. Endogenous peroxidases were inhibited with 0.5% H₂O₂ in distilled water for 15 minutes at room temperature. Thereafter samples were washed ten times with tap water. Antigen retrieval was performed by incubating the slides in Tris-EDTA pH 9.0 for 30 minutes using a steamer. Afterwards slides were allowed to cool down for 30 minutes. Incubation in 1.5% normal goat serum (Sigma Aldrich) in PBS for 30 minutes was used to minimize nonspecific antibody binding. Sections were incubated with the primary antibody at a dilution of 1:500 in PBS overnight at 4°C. On the next day, sections were washed with PBS and incubated with the secondary antibody (BrightVision Poly-HRP-anti-mouse, ImmunoLogic, Duiven, The Netherlands) for 30 minutes at room temperature. Subsequently, sections were washed in PBS and incubated in DAB-Solution (Quanto Richard Allan Scientific, TA-125-QHDX) for 5 minutes. Thereafter, sections were washed in distilled water and counterstained for 3 minutes with Mayer's Haemalaun, washed with tap water for 10 minutes, dehydrated, and mounted with DPX (Sigma Aldrich).

Table 1
RT-qPCR assay details.

Gene symbol	NCBI accession number	Primer sequence (5' – 3')		Amplicon size (bp)	PCR amplification efficiency (E)	Reference
		Forward	Reverse			
IGF1	XM_005606469.3; XM_005606470.3; XM_005606471.3; XM_005606472.3	CGCTCTTCAGTTCGTGTGT	AGTACATCTCCAGCCTCCTC	146	0,95	-
IGF1R	XM_023651179.1	GTGGACTGATCCTGTGTCTTC	TAACCAACCCCTCCCACTATCA	113	0,99	-
IGF2	NM_001114539.2; XM_023653770.1; XM_023653771.1; XM_023653772.1; XM_023653773.1; XM_023653774.1; XM_023653775.1; XM_023653776.1; XM_023653777.1; XM_023653778.1; XM_023653779.1; XM_023653780.1; XM_023653781.1; XM_023653782.1; XM_023653783.1	GATACCCTCCAGTTGTCTGTG	AACAGCACTCTTCCACGATG	100	1	-
IGF2R	XM_005608119.3; XM_023632974.1	GACCAGCACTTCAGTAGGAAAG	CACCTAGAGGATGAGGCAAACA	98	1,01	-
PRLR	XM_001500104.4	CATGGTATCCTGCATCCTCC	CAGGGCACTCAGTAGTCTTC	103	0,99	-
ACTB	NM_001081838.1	CGGGACCTGACGGACTA	CCTTGATGTACGCACGATT	94	0,91	Scarlet et al., 2015
GAPDH	NM_001163856.1, XM_008513936.1	GGCAAGTTCATGGCACAGT	CACAACATATTACGACCAGCAT	129	0,9	Scarlet et al., 2015
PSMB4	XM_001492317.4, XM_005610132.1, XM_008515015.1, XM_005613704.1	CTTGGTGTAGCCTATGAAGCCC	CCAGAATTCTCGCAGCAGAG	82	0,93	Scarlet et al., 2015
SNRPD3	XM_001489060.4, XM_008511652.1	ACGCACCTATGTAAAGAGCATG	CACGTCCCATTCACGTC	120	0,99	Scarlet et al., 2015

The slides were evaluated with an Axio Imager Z2 light microscope (Carl Zeiss, Jena, Germany) at 50x (NA 0.16) and 200x (NA 0.8) magnification connected with the software Zeiss Zen blue edition (Carl Zeiss, Munich, Germany), and images were recorded in tiff format. In amniotic tissue, five randomly chosen sections were examined at 200x (NA 0.8) magnification. The chorioallantois was differentiated into the microvilli and endothelial region. From both areas, 10 sections were evaluated. For quantitative immunohistology, sections of chorioallantois, both uterine horns and the amnion were analyzed using the open-source image processing software Fiji [40]. From all fields, the non-tissue regions were excluded to obtain the actual total tissue area. After assessment of the fields, a mean value was calculated and expressed in μm^2 and % of the stained tissue, respectively.

2.7. Statistical analysis

Statistical analysis was performed with the IBM SPSS statistics software (version 29; IBM, Armonk, NY, USA). Parameters determined repeatedly over time (follicular size, hormone concentrations) were analysed by GLM (general linear model) repeated measures ANOVA with time as within subject factor and group as between subject factor. Parameters determined by qPCR or immunohistochemistry at foaling but analyzed in different placental tissues were compared by GLM univariate analysis with both group and localization as fixed effects. Data obtained at one time point only such as day of foaling or gestation length were compared between groups by Wilcoxon signed rank test. For all comparisons, a p-value <0.05 was considered significant. Data are given as mean and standard error (SEM).

3. Results

3.1. Foaling and postpartum ovulation

In the two years of the study, five mares ovulated within 15 days after foaling and nine mares did not ovulate within 15 days after foaling. Two mares ovulated within 15 days after foaling in both years and one mare ovulated within 15 days after foaling in the first year of the study. Out of the five OV mares, one ovulated on day 11, two on day 12 and one each on days 14 and 15 after foaling.

Mares which did not ovulate until day 15 postpartum (Group NOV) foaled earlier in the year (range: January 29th to March 25th) than mares that did ovulate (range: February 9th to April 25th) during that time (p<0.05; Table 2). Gestation length, placental weight, placental surface, and time from foaling until shedding of the placenta did not differ between groups.

3.2. Follicular growth

Already 15 days before foaling, follicles with a size larger than 1 cm in diameter were present on both ovaries in all mares. The number of follicles with a diameter between one and 2.5 cm increased from 15 days before to 5 days after foaling (p<0.05; Fig. 1a,b). The diameter of the largest follicle increased markedly after foaling in mares of both groups. On the left ovary, NOV mares had a larger follicle than OV mares from

day 5 after foaling (day p<0.001, group p<0.05, day x group p<0.05). In NOV mares, size of the follicle continued to grow and in OV mares, follicle size decreased. Ovulation in mares that ovulated within 15 days after foaling always occurred on the right ovary. On the day before ovulation, the largest follicle had a size of 4.83 ± 0.3 cm. On this ovary, size of the largest follicle increased in both groups from the day of foaling (day p<0.001, group p<0.05, day x group p<0.01). The dominant follicle in NOV mares was smaller than in OV mares over the entire observation period.

3.3. Endocrine analyses

The average concentration of LH was constantly low before foaling and increased continuously after foaling in all mares irrespective of group (Figure 2a; p<0.001). This increase was more pronounced in mares that ovulated within 15 days postpartum than in mares that did not ovulate (day x group p<0.001). The concentration of FSH increased transiently one day after foaling in mares of both groups and subsequently decreased again (Figure 2b; p<0.001). From day 12 after foaling onwards, FSH concentration increased again in mares that ovulated within 15 days postpartum. Activin A concentration increased before foaling, peaked on the day of foaling and decreased rapidly thereafter (Figure 2c; p<0.001). Activin A concentration did not differ between OV and NOV mares.

The concentration of IGF-1 in plasma increased during the last days before foaling in mares of both groups (p<0.001, Fig. 2d). An increase in plasma IGF-2 concentration was less pronounced than in IGF-1 (p<0.001, Fig. 2e). Plasma prolactin concentration increased rapidly during the last five days before foaling, peaked on day one after foaling and had returned to baseline values on day 3 after foaling (Figure 2f; p<0.001).

3.4. Quantitative mRNA analysis

The IGF-1 mRNA abundance was greater in the allantochorion but decreased in the amnion of OV compared to NOV mares (group p=0.01, localization x group p<0.01; Fig. 3a). Abundance of IGF-2 mRNA differed neither between OV and NOV mares nor between the amnion and allantochorion (Fig. 3b). The IGF-1 receptor mRNA was more abundant in the allantochorion than in the amnion irrespective of mare group (p<0.001; Fig. 3c) whereas IGF-2 receptor mRNA differed neither between amnion and allantochorion nor between OV and NOV mares (Fig. 3d). The mRNA abundance for the prolactin receptor differed neither between groups OV and NOV nor between the amnion and allantochorion (Fig. 3e).

3.5. Immunohistochemistry

The IGF-1 protein was expressed in the allantochorion and amnion with no differences between mare groups. Expression of IGF-1 protein tended to be more pronounced in the allantochorion than in the amnion (p=0.05; Fig. 4a, Fig. 5). The prolactin receptor was expressed in the amnion and allantochorion with most pronounced expression in the allantochorionic microvilli (p<0.001; Figure 4b; Fig. 5). Prolactin receptor protein expression in the allantochorion and amnion did not differ between OV and NOV mares.

4. Discussion

Results of our study indicate that growth of ovarian tertiary follicles in mares is re-initiated well before foaling. The number of antral follicles with a diameter of more than 10 mm increased during the last two weeks before foaling and at the same time the largest follicle detectable by ultrasound doubled its diameter. The rapid resumption of ovulatory cycles in postpartum mares is thus enabled by follicular growth occurring already in late pregnancy and thus at a time when mares are still

Table 2
Foaling date, gestation length, placental weight, placental surface, and time until placental shedding in mares ovulating within (OV) or after 15 days postpartum (NOV; n=9).

Group	OV (n=5)	NOV (n=9)
Foaling date (day of the year and range)	84±29 (40-115) a	50±20 (25-85) b
Gestation length (days)	332±7	338±9
Placental weight (kg)	4.2±0.8	4.0±0.5
Placental surface (m ²)	1.3±0.2	1.3±0.1
Time to placental shedding (min)	53±26	75±37

a,b p<0.05

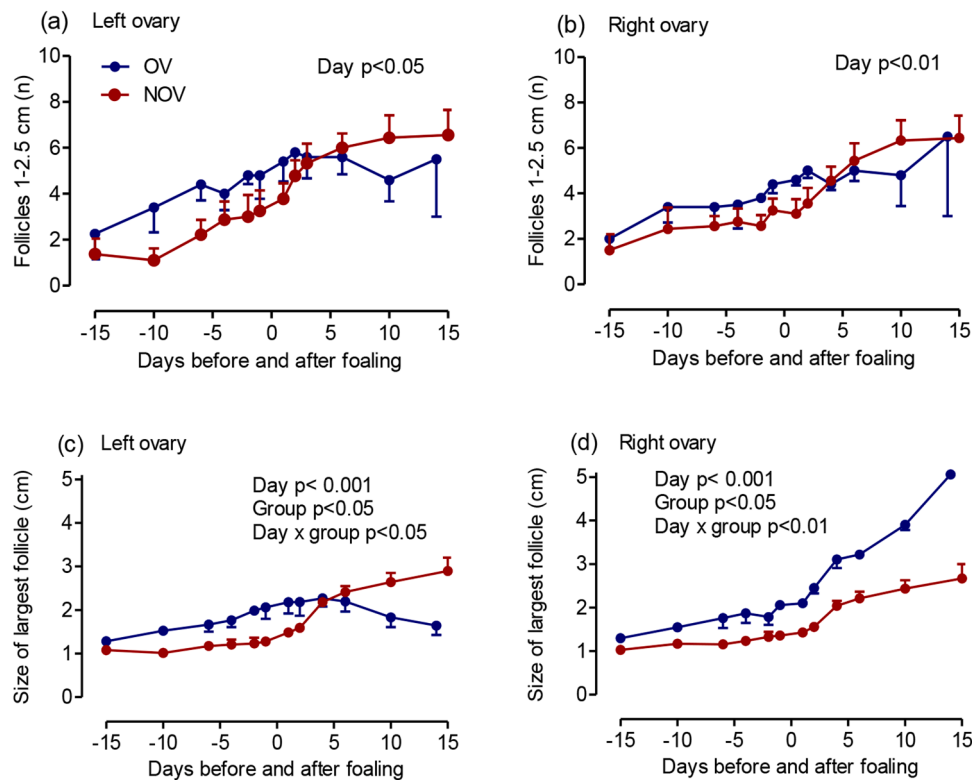


Fig. 1. (a,b) Number of follicles with a diameter of 1-2.5 cm and (c,d) size of the largest follicle on the (a,c) left and (b,d) right ovary from 15 days before to 15 days after foaling in mares ovulating until day 15 days after foaling (OV, $n=5$) and not ovulating until day 15 days after foaling (NOV, $n=9$). Significant differences are indicated in the figures.

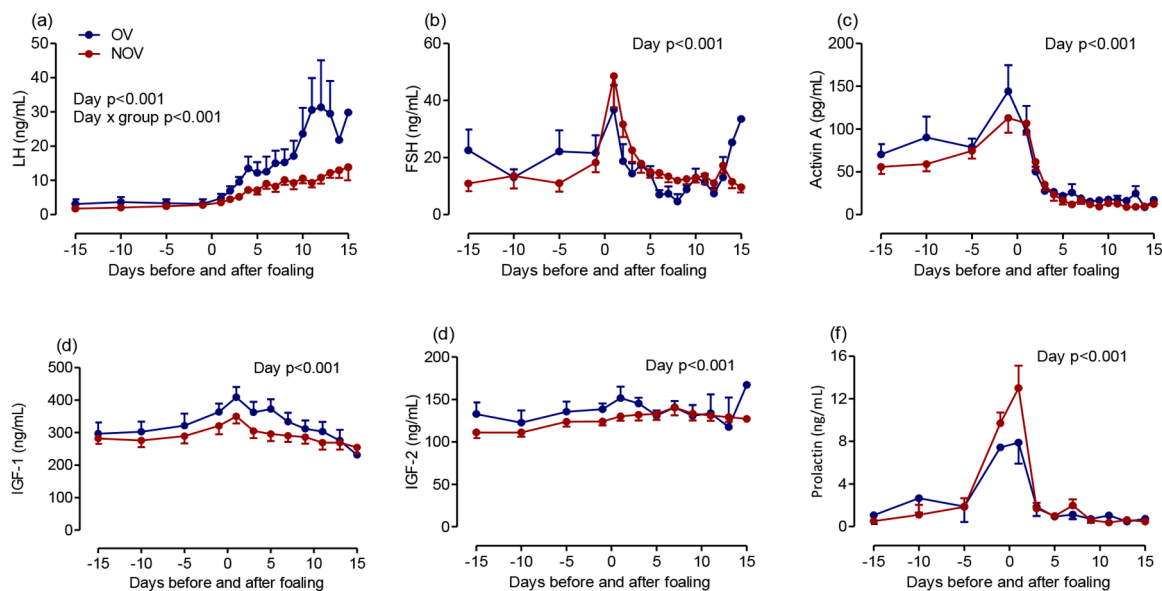


Fig. 2. Concentration of (a) LH, (b) FSH, (c) activin A, (d) IGF-1, (e) IGF-2 and (f) prolactin in serum of mares ovulating until day 15 days after foaling (OV, $n=5$) and not ovulating until day 15 days after foaling (NOV, $n=9$) from 15 days before to 15 days after foaling. Significant differences are indicated in the figures.

under the influence of progestogens derived from the fetoplacental unit. Follicle diameter increased further after foaling. Already before foaling, follicular growth was more pronounced in mares that ovulated within 15 days postpartum than in mares with delayed ovulation. We have no explanation for the fact that all detected ovulations occurred on the right ovary, and this may be a coincidence due to a relatively small number of animals where ovulations occurred.

To the best of our knowledge, follicle growth has not been analyzed

towards the end of equine gestation so far. Follicular waves continue in early equine pregnancy with irregular minor and major waves [41] but from day 70 of gestation onwards follicular activity decreases with follicles no longer exceeding a diameter of 20 mm [42]. Similar observations have been reported from cows, although follicular waves persist longer in pregnant cows than mares [43].

In agreement with the rapidly increasing growth of follicles after foaling, there was a transient increase in FSH release until five days

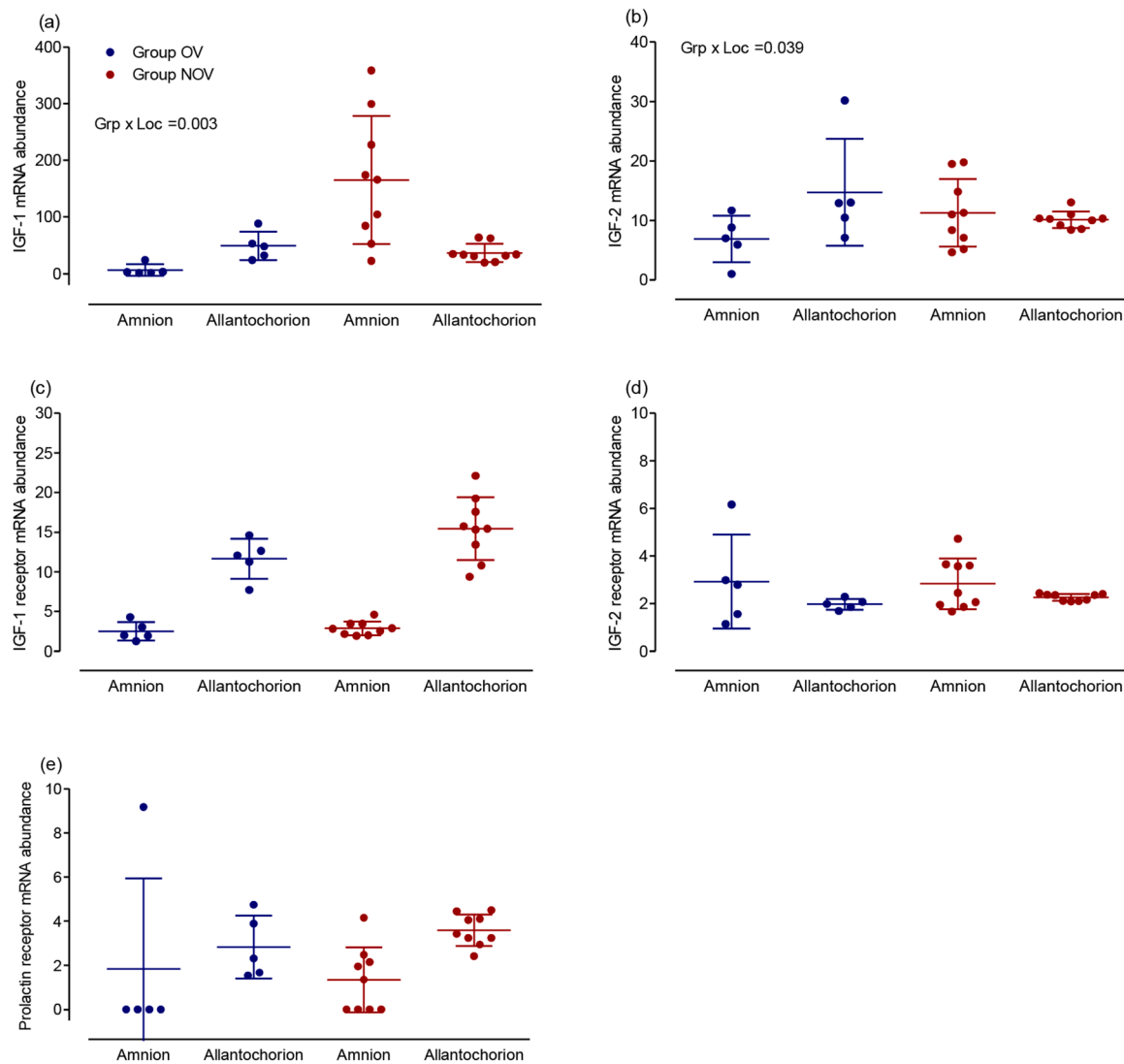


Fig. 3. Abundance of (a) IGF-1 mRNA, (b) IGF-2 mRNA, (c) IGF-1 receptor mRNA (d) IGF-2 receptor mRNA and (e) prolactin receptor mRNA at foaling in the amnion and the pregnant uterine horn allantochorion of mares ovulating until day 15 days after foaling (Group OV, n=5) and not ovulating until day 15 days after foaling (Group NOV, n=9), note different scale of y-axis.

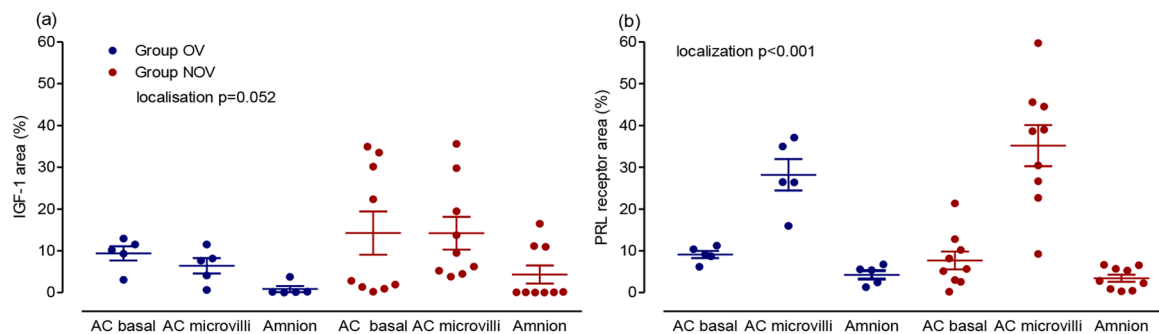


Fig. 4. Area (%) of cells stained positive for (a) IGF-1 and (b) the prolactin receptor in the endothelial and microvilli areas of the allantochorion (AC) and amnion of mares ovulating until day 15 days after foaling (Group OV, n=5) and mares not ovulating until day 15 days after foaling (Group NOV, n=9).

postpartum and a continuous rise in mean LH concentration during the first two weeks after foaling. The marked FSH release agrees with previous studies [44,45]. As in our study, serum FSH concentration early postpartum did not differ between mares with an early or a delayed first postpartum ovulation [45]. In mares with a diameter of the largest follicle less than 20 mm, a delayed decrease in FSH concentration has

been reported [45] suggesting reduced negative feedback on FSH release. Because all mares in our study had larger follicles, reduced negative feedback on FSH release was not evident. In mares of both groups, plasma activin A concentration peaked one day before foaling and then decreased. Activin promotes the release of FSH [46,33] and thus most likely stimulates the postpartum rise in plasma FSH

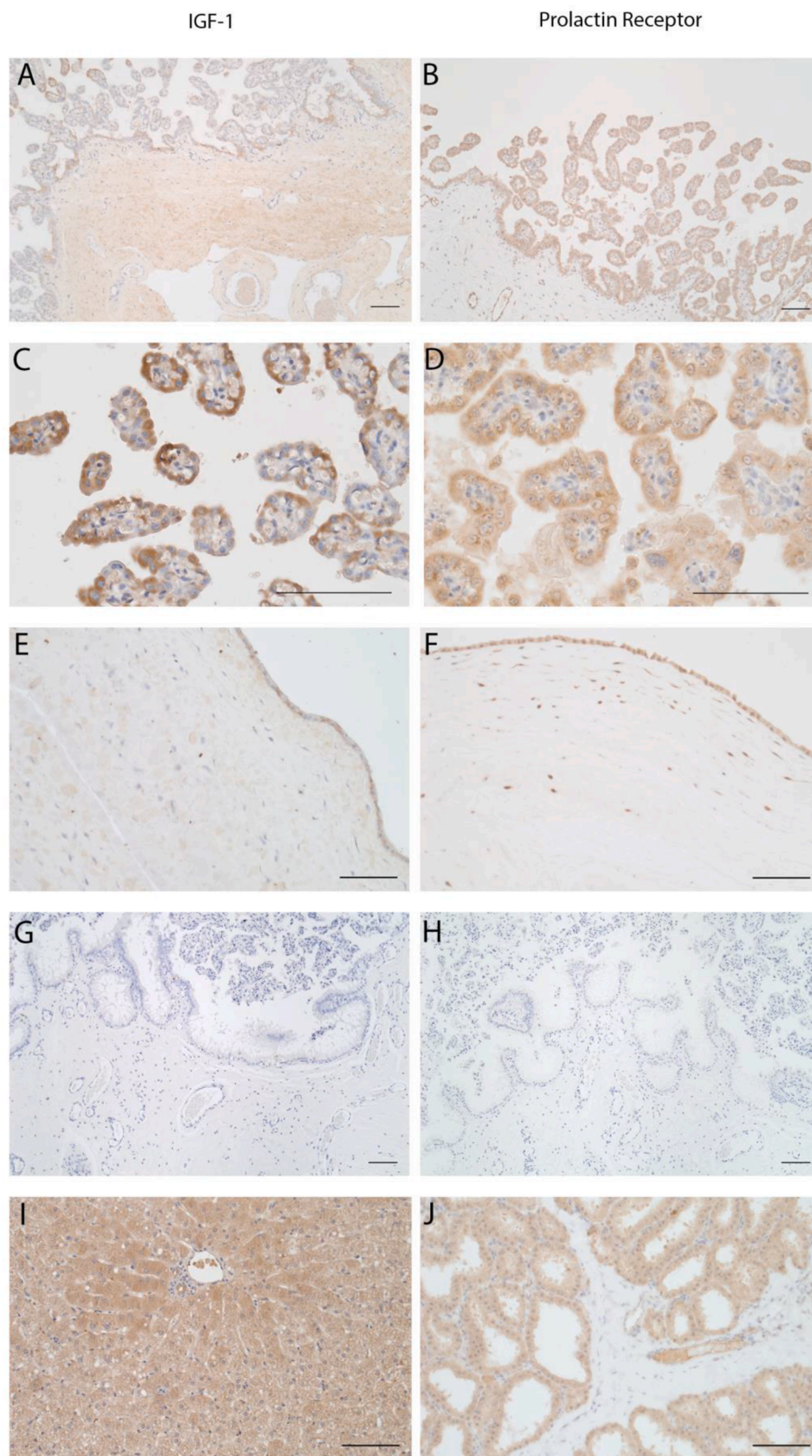


Fig. 5. Immunohistochemical staining for IGF-1 (A, C, E, G, I) and prolactin receptor (B, D, F, H, J) in the allantochorion (A, B, C, D) and amnion (E, F). Negative isotop control of IGF-1 (G) and prolactin receptor (H) in the allantochorion and positive isotope control of IGF-1 (I) in the equine liver and prolactin receptor (J) in the equine mamma. In all figures, scale bar=100 μ m.

concentration.

The increase in average serum LH concentration was more pronounced in mares that ovulated within 15 days after foaling compared to mares with delayed ovulation. As expected, in the mares with early ovulation, also growth of the largest and presumably dominant follicle was more pronounced. This is in agreement with positive feedback between estradiol synthesis increasing with follicle size and LH release. Mares that did not ovulate within 15 days postpartum had foaled on average one month earlier in the year than ovulating mares. Differences in follicular growth and ovulation rate between the mare groups are most likely caused by the increased stimulatory effects of daylight in the mares that foaled later in the year. Mares foaling early in the year are exposed to the contradictory effects of parturition (a stimulatory effect on ovulation) and the anovulatory season (a suppressive effect on ovulation). A delayed onset of foal heat in mares foaling earlier in the year has been reported previously [3] and is due to photoperiod-regulated, reduced LH release in winter [47]. In the majority of horse mares [1–4] but only in a small percentage of pony mares [48], the stimulatory endocrine effect of parturition that was also investigated in the present study is sufficient to overcome the effects of season. The present results demonstrate that Haflinger mares are, however, more similar to ponies in this regard. Light programs [49] or light masks emitting blue LED light [50] initiated approximately 60 days before foaling could be used to advance postpartum ovulations in such mares.

Confirming several previous studies [12–16,49], concentrations of IGF-1 and prolactin in plasma of mares increased before foaling and peaked on day 1 after foaling. Based on mRNA and protein expression, our present data indicate that IGF-1, at least in part, is synthesized by the allantochorion and amnion of late pregnant mares until foaling. Together with the antepartum increase in plasma IGF-1 concentration, also placental expression of IGF-1 may increase. Although placental IGF-1 mRNA abundance was lower in mares ovulating within 15 days postpartum than in mares with delayed ovulation, neither IGF-1 protein expression nor expression of the IGF-1 receptor or concentration of IGF-1 in plasma differed between groups.

During gestation, prolactin release in the mare is inhibited by endogenous opioids but this inhibition ceases after foaling and allows the pronounced increase in prolactin release [51]. Prolactin alone appears to be without direct effects on follicular growth in mares [16] but together with IGF-1 has been suggested to stimulate postpartum follicle growth [12]. The present findings with regard to prolactin receptor protein expression and mRNA abundance in the allantochorion of mares suggest a responsiveness of placental IGF-1 synthesis to prolactin.

A positive effect of IGF-1 on gonadotropin synthesis and release and ovarian activity has been demonstrated in several species, including pigs [27,29] and cattle [52]. In dairy cows, reduced IGF-1 release mainly reflects energy balance [53,54]. In sows, IGF-1 release is stimulated by growth hormone during a state of nutritional energy deficiency allowing sows to compensate a deficient metabolic state much better than cows [54]. Concentrations of IGF-1 are reduced in mares exposed to restrictive feeding [55]. With adequate feeding as in the present study, postpartum mares meet the metabolic demand of lactation without going through a phase of negative energy balance [3,12]. The difference between groups in time until first ovulation is therefore more likely an effect of season than energy balance and IGF-1 concentration. Concomitant with increased IGF-1 release in early postpartum mares, there is at the same time also a decrease in plasma leptin concentrations [3,56]. Reduced leptin concentrations may stimulate feed intake in early lactating mares, resulting in adequate energy uptake.

There was only a small increase in plasma IGF-2 concentration at foaling and no differences between groups in plasma IGF-2 concentration, placenta IGF-2 protein expression and mRNA abundance. In sheep, as in the mares of the present study, the receptors for IGF-1 and IGF-2 are strongly expressed in the placenta. A paracrine action of placental IGF protein on placental function and fetal development has therefore been

suggested in this species [54]. Such effects are mediated, however, predominantly via the IGF-1 receptor and the IGF-2 receptor has been suggested to function at least in part as an IGF-2-specific binding protein [57]. There is only little evidence to support a signaling function for growth-promoting effects of IGF-1 and IGF-2 [58].

In conclusion, ovarian follicular growth in mares is initiated already before foaling, enabling mares to resume cyclic ovarian activity rapidly after foaling. Development of follicles to a preovulatory stage occurs, however, with a marked increase in first FSH and then LH release after foaling. This is reduced in mares foaling early in the year and may thus suppress ovulation. On the other hand, resumption of ovulatory cycles may be enhanced by prolactin-stimulated IGF-1 synthesized at least in part by the placenta until foaling.

CRedit authorship contribution statement

Maria Melchert: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Jörg Aurich:** Writing – original draft, Resources, Data curation. **Reinhard Ertl:** Methodology, Investigation. **Ursula Reichart:** Methodology, Investigation. **Ingrid Walter:** Methodology, Investigation. **Camille Gautier:** Methodology, Investigation. **Martim Kaps:** Investigation. **Christine Aurich:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors do not have any interest to declare.

Acknowledgments

The authors are grateful to Julia Maderner for expert help with the endocrine analyses and Claudia Höchsmann for preparing the histology slides. This research was supported using resources of the VetCore Facility (Imaging) of the University of Veterinary Medicine Vienna.

References

- [1] Bain AM. Estrus and infertility of the Thoroughbred mare in Australia. *J Am Vet Med Assoc* 1957;131:179–85.
- [2] Nagy P, Huszenicza G, Juhasz J, Kulcsar M, Solti L, Reiczgel J, Abavary K. Factors influencing ovarian activity and sexual behavior of postpartum mares under farm conditions. *Theriogenology* 1998;50:1109–19. [https://doi.org/10.1016/S0093-691X\(98\)00212-X](https://doi.org/10.1016/S0093-691X(98)00212-X).
- [3] Heidler B, Aurich J, Pohl W, Aurich C. Body weight of mares and foals, estrous cycles and plasma glucose concentration in lactating and non-lactating Lipizzaner mares. *Theriogenology* 2004;61:883–93. [https://doi.org/10.1016/S0093-691X\(03\)00279-6](https://doi.org/10.1016/S0093-691X(03)00279-6).
- [4] Kuhl J, Aurich JE, Wulf M, Hurtienne A, Schweigert FJ, Aurich C. Effects of oral supplementation with β -carotene on concentrations of β -carotene, vitamin A and α -tocopherol in plasma, colostrum and milk of mares and plasma of their foals and on fertility in mares. *J Anim Physiol Anim Nutr* 2012;96:376–84. <https://doi.org/10.1111/j.1439-0396.2011.01150.x>.
- [5] Giudice LC, Mark SP, Irwin JC. Paracrine actions of insulin-like growth factors and IGF binding protein-1 in non-pregnant human endometrium and at the decidual-trophoblast interface. *J Reprod Immunol* 1998;39:133–48. [https://doi.org/10.1016/S0165-0378\(98\)00018-7](https://doi.org/10.1016/S0165-0378(98)00018-7).
- [6] Han VKM, Carter AM. Spatial and temporal patterns of expression of messenger RNA for insulin-like growth factors and their binding proteins in the placenta of man and laboratory animals. *Placenta* 2000;21(4):289–305. <https://doi.org/10.1053/plac.1999.0498>.
- [7] Arai KY, Tanaka Y, Taniyama H, Tsunoda N, Nambo Y, Nagamine N, Watanabe G, Taya K. Expression of inhibins, activins, insulin-like growth factor-I and steroidogenic enzymes in the equine placenta. *Domestic Anim Endocrinol* 2006;31:19–34. <https://doi.org/10.1016/j.domaniend.2005.09.005>.
- [8] Spicer LJ, Tucker KE, Henderson KA, Duby RT. Concentrations of insulin-like growth factor-I in follicular fluid and blood plasma of mares during early and late oestrus. *Anim Reprod Sci* 1991;25:57–65. [https://doi.org/10.1016/0378-4320\(91\)90008-N](https://doi.org/10.1016/0378-4320(91)90008-N).
- [9] Bridges TS, Davidson TR, Chamberlain CS, Geisert RD, Spicer LJ. Changes in follicular fluid steroids, insulin-like growth factors (IGF) and IGF-binding protein concentration, and proteolytic activity during equine follicular development. *J Anim Sci* 2002;80:179–90. <https://doi.org/10.2527/2002.801179x>.

- [10] Ginther OJ, Meira C, Beg MA, Bergfelt DR. Follicle and endocrine dynamics during experimental follicle deviation in mares. *Biol Reprod* 2002;67(3):862–7. <https://doi.org/10.1095/biolreprod.102.004309>.
- [11] Donadeu FX, Ginther OJ. Changes in Concentrations of Follicular Fluid Factors During Follicle Selection in Mares. *Biol Reprod* 2002;66(4):1111–8. <https://doi.org/10.1095/biolreprod66.4.1111>.
- [12] Heidler B, Parvizi N, Sauerwein H, Bruckmaier RM, Heintges U, Aurich JE, Aurich C. Effects of lactation on metabolic and reproductive hormones in Lipizzaner mares. *Domestic Anim Endocrinol* 2003;25:47–59. [https://doi.org/10.1016/S0739-7240\(03\)00044-4](https://doi.org/10.1016/S0739-7240(03)00044-4).
- [13] Nett TM, Holtan DW, Estergreen VL. Levels of LH, prolactin and oestrogens in the serum of post-partum mares. *J Reprod Fertil Suppl* 1975;23:201–6.
- [14] Lotherop CD, Henton JE, Cole BB, Nolan HL. Prolactin response to thyrotrophin-releasing hormone stimulation in normal and agalactic mares. *J Reprod Fertil Suppl* 1987;35:277–80.
- [15] Worthy K, Colquhoun K, Escuret R, Dunlop M, Renton JP, Douglas TA. Plasma prolactin concentrations in non-pregnant mares at different times of the year and in relation to events in the cycle. *J Reprod Fertil Suppl* 1987;35:269–76. 1987.
- [16] Neuschaefer A, Bracher V, Allen WR. Prolactin secretion in lactating mares before and after treatment with bromocriptine. *J Reprod Fertil Suppl* 1991;44:551–9.
- [17] Ireland FA, Loch WE, Worthy K, Anthony RV. Effects of bromocriptine and perphenazine on prolactin and progesterone concentrations in pregnant pony mares during late gestation. *J Reprod Fertil* 1991;92:179–86. <https://doi.org/10.1530/jrf.0.0920179>.
- [18] Schams D, Graf F, Graule B, Abele M, Prokopp S. Hormonal changes during lactation in cows of three different breeds. *Livestock Prod Sci* 1991;27:285–96. [https://doi.org/10.1016/0301-6226\(91\)90124-9](https://doi.org/10.1016/0301-6226(91)90124-9).
- [19] Block SS, Butler RW, Ehrhardt RA, Bell AW, van Amburgh ME, YRI Boisclair. Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. *J Endocrinol* 2001;171:339–48. <https://doi.org/10.1677/joe.0.1710339>.
- [20] Wathes DC, Perks CM, Davis AJ, Denning-Kendall PA. Regulation of insulin-like growth factor I and progesterone synthesis by insulin and growth hormone in the bovine ovary. *Biol Reprod* 1995;53:882–9. <https://doi.org/10.1095/biolreprod53.4.882>.
- [21] Gong JG, Baxter G, Bramley TA, Webb R. Enhancement of ovarian follicle development in heifers by treatment with recombinant bovine somatotrophin: a dose-response study. *J Reprod Fertil* 1997;110:91–7. <https://doi.org/10.1530/jrf.0.1100091>.
- [22] Chase CC, Kirby CJ, Hammond AC, Olson TA, Lucy MC. Patterns of ovarian growth and development in cattle with a growth hormone receptor deficiency. *J Anim Sci* 1998;76:212–9. <https://doi.org/10.2527/1998.761212x>.
- [23] Gong JG, McBride D, Bramley TA, Webb R. Effects of recombinant bovine somatotrophin, insulin-like growth factor I and insulin on the proliferation of bovine granulosa cells in vitro. *J Endocrinol* 1993;139:67–75. <https://doi.org/10.1677/joe.0.1390067>.
- [24] Schams D, Kraetzl WD, Brem G, Graf F. Secretory pattern of metabolic hormones in the lactating sow. *Exp Clin Endocrinol* 1994;102:439–47. <https://doi.org/10.1055/s-0029-1211316>.
- [25] Giovoni N, Parmeggiani A, Galeati G, Penazzi P, De Iasio R, Pagotto U, Pasquali R, Tamanini C, Seren E. Acyl ghrelin and metabolic hormones in pregnant and lactating sows. *Reprod Domest Anim* 2007;42:39–43. <https://doi.org/10.1111/j.1439-0531.2006.00722.x>.
- [26] Kraetzl WD, Zimmer C, Schneider D, Schams D. Secretion pattern of growth hormone, prolactin, insulin and insulin-like growth factor-1 in the periparturient sow depending on the metabolic state during lactation. *Animal Science* 1998;67(2):339–47. <https://doi.org/10.1017/S1357729800010110>.
- [27] van den Brand H, Prunier A, Soede NM, Kemp B. In primiparous sows, plasma insulin-like growth factor-I can be affected by lactational feed intake and dietary energy source and is associated with luteinizing hormone. *Reprod Nutr Dev* 2001;41:27–39. <https://doi.org/10.1051/rnd:2001109>.
- [28] Ferguson EM, Ashworth CJ, Edwards SA, Hawkins N, Hepburn N, Hunter MG. Effect of different nutritional regimens before ovulation on plasma concentrations of metabolic and reproductive hormones and oocyte maturation in gilts. *Reproduction* 2003;126:61–71. <https://doi.org/10.1530/rep.0.1260061>.
- [29] Costermans NGJ, Teerds KJ, Middelkoop A, Roelen BAJ, Schoevers EJ, van Tol HTA, Laurensen B, Koopmanschap RE, Zhao Y, Blokland M, van Tricht F, Zak L, Keijer J, Kemp B, Soede NM. Consequences of negative energy balance on follicular development and oocyte quality in primiparous sows. *Biol Reprod* 2020;102:388–98. <https://doi.org/10.1093/biolre/iz175>.
- [30] Han T, Björkman S, Soede NM, Oliviero C, Peltoniemi OAT. IGF-1 concentration patterns and their relationship with follicle development after weaning in young sows fed different pre-mating diets. *Animal* 2020;14:1493–501. <https://doi.org/10.1017/S1751731120000063>.
- [31] Gastal EL, MO Gastal, Bergfelt DR, Ginther OJ. Role of diameter differences among follicles in selection of a future dominant follicle in mares. *Biol Reprod* 1997;57:1320–7. <https://doi.org/10.1095/biolreprod57.6.1320>.
- [32] Nett TM, Pickett BW, Squires EL. Effects of Equimate (ICI-81008) on levels of luteinizing hormone, follicle-stimulating hormone and progesterone during the estrous cycle of the mare. *J Anim Sci* 1979;48:69–75. <https://doi.org/10.2527/jas1979.48169x>.
- [33] Dhakal P, Tsunoda N, Nambo Y, Taniyama H, Nagaoka K, Watanabe G, Taya K. Circulating activin A during equine gestation and immunolocalization of its receptors system in utero-placental tissues and fetal gonads. *J Equine Sci* 2021;32:39–48. <https://doi.org/10.1294/jes.32.39>.
- [34] Allen WR, Wilsher S, Turnbull C, Stewart F, Ousey J, Rossdale PD. Influence of maternal size on placental, fetal and postnatal growth in the horse. I. Development in utero. *Reproduction* 2002;123:445–53. <https://doi.org/10.1530/rep.0.1230445>.
- [35] Pazinato FM, da Rosa Curcio B, Varela AS, Dahl Corcini C, Gervini Wendt C, Moreira F, Alves Schmit R, Wayne Nogueira CE. Immunolocalization of leptin and its receptor (ObR-b) in equine placenta at term and plasma level measurement in the late gestation. *J Equine Vet Sci* 2019;78:1–5. <https://doi.org/10.1016/j.jevs.2019.03.008>.
- [36] Scarlet D, Ertl R, Aurich C, Steinborn R. The orthology clause in the next generation sequencing era: Novel reference genes identified by RNA-seq in humans improve normalization of neonatal equine ovary RTqPCR data. *PLoS One* 2015;10:142122. <https://doi.org/10.1371/journal.pone.0142122>.
- [37] Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: A miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Molec Biol* 2012;80:75–84. <https://doi.org/10.1007/s11103-012-9885-2>.
- [38] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 2001;25:402–8. <https://doi.org/10.1006/meth.2001.1262>.
- [39] Beckelmann J, Budik S, Helmreich M, Palm F, Walter I, Aurich C. Sex-dependent insulin like growth factor-1 expression in preattachment equine embryos. *Theriogenology* 2013;79:193–9. <https://doi.org/10.1016/j.theriogenology.2012.10.004>.
- [40] Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. *Nat Meth* 2012;9:676–82. <https://doi.org/10.1038/nmeth.2019>.
- [41] Ginther OJ, Bergfelt DR. Associations between FSH concentrations and major and minor follicular waves in pregnant mares. *Theriogenology* 1992;38:807–21. [https://doi.org/10.1016/0093-691X\(92\)90157-M](https://doi.org/10.1016/0093-691X(92)90157-M). 1992.
- [42] Squires EL, Garcia MC, Ginther OJ. Effects of pregnancy and hysterectomy on the ovaries of pony. *J Anim Sci* 1974;38:823–30. <https://doi.org/10.2527/jas1974.384823x>.
- [43] Ginther OJ, Kot K, Kulick LJ, Martin S, Wiltbank MC. Relationships between FSH and ovarian follicular waves during the last six months of pregnancy in cattle. *J Reprod Fert* 1996;108:271–9. <https://doi.org/10.1530/jrf.0.1080271>.
- [44] Turner DD, Garcia MC, Miller KF, Holtan DW, Ginther OJ. FSH and LH concentrations in periparturient mares. *J Reprod Fertil Suppl* 1979;27:547–53.
- [45] Ginther OJ, Baucus KL, Bergfelt DR. Follicular and FSH responses to parturition during the anovulatory season in mares. *Theriogenology* 1994;41:613–27. [https://doi.org/10.1016/0093-691X\(94\)90172-F](https://doi.org/10.1016/0093-691X(94)90172-F).
- [46] Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin R. Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. *Nature* 1986;321:779–82. <https://doi.org/10.1038/321779a0>.
- [47] Palmer E, Driancourt MA. Some interactions of season of foaling, photoperiod, and ovarian activity in the equine. *Livestock Prod Sci* 1983;10:197–210.
- [48] Hodge SL, Kreider JL, Potter GD, Harms PG, Fleeger JL. Influence of photoperiod on the pregnant and postpartum mare. *Am J Vet Res* 1982;43:1752–5.
- [49] Lutzer A, Nagel C, Murphy BA, Aurich J, Wulf M, Gautier C, Aurich C. Effects of blue monochromatic light directed at one eye of pregnant horse mares on gestation, parturition and foal maturity. *Domest Anim Endocrinol* 2022;78:106675. <https://doi.org/10.1016/j.domaniend.2021.106675>.
- [50] Hess-Dudan F, Vacher PY, Bruckmaier RM, Weishaupt MA, Burger D, Blum JW. Immunoreactive insulin-like growth factor I and insulin in blood plasma and milk of mares and in blood plasma of foals. *Equine Vet J* 1994;26(2):134–9. 1994.
- [51] Aurich C, Gerlach T, Aurich JE, Parvizi N. Seasonal variation and opioidergic regulation of growth hormone release in cyclic, ovariectomized, and pregnant pony mares. *Biol Reprod* 1999;61:1575–80. <https://doi.org/10.1095/biolreprod61.6.1575>.
- [52] Armstrong DG, McEvoy TG, Baxter G, Robinson JJ, Hogg CO, Woad KJ, Webb R, Sinclair KD. Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. *Biol Reprod* 2001;64:1624–32. <https://doi.org/10.1095/biolreprod64.6.1624>.
- [53] Wathes DC, Cheng Z, Salavati M, Buggiotti L, Takeda H, Tang L, Becker F, Ingvarsen KI, Ferris C, Hostens M, Crowe MA. Relationships between metabolic profiles and gene expression in liver and leukocytes of dairy cows in early lactation. *J Dairy Sci* 2021;104(3):3596–616. <https://doi.org/10.3168/jds.2020-19165>.
- [54] Kraetzl WD, Zimmer C, Schneider D, Schams D. Secretion pattern of growth hormone, prolactin, insulin and insulin-like growth factor-1 in the periparturient sow depending on the metabolic state during lactation. *Animal Science* 1998;67(2):339–47. <https://doi.org/10.1017/S1357729800010110>.
- [55] Gentry LR, Thompson DL, Gentry GT, Davis KA, Godke RA, Cartmill JA. The relationship between body condition, leptin, and reproductive and hormonal characteristics of mares during the seasonal anovulatory period. *J Anim Sci* 2002;80:2695–703. [https://doi.org/10.1016/S0093-691X\(02\)00841-5](https://doi.org/10.1016/S0093-691X(02)00841-5).
- [56] Arfuso F, Giannetto C, Bazzano M, Assenza A, Piccione G. Physiological Correlation between Hypothalamic–Pituitary–Adrenal Axis, Leptin, UCP1 and Lipid Panel in

- Mares during Late Pregnancy and Early Postpartum Period. *Animals* 2021;11(7): 2051. <https://doi.org/10.3390/ani11072051>.
- [57] Gelato MC, Rutherford C, Stark RI, Daniel SS. The insulin-like growth factor II/ mannose-6-phosphate receptor is present in foetal and maternal sheep serum. *Endocrinology* 1989;124:2935–43. <https://doi.org/10.1210/endo-124-6-2935>.
- [58] D'Ercole AJ. Insulin-like growth factors and their receptors in growth. *Endocrinol Metab Clin North Am* 1996;25:573–90. [https://doi.org/10.1016/S0889-8529\(05\)70341-8](https://doi.org/10.1016/S0889-8529(05)70341-8).