Expression of Steroidogenic Enzyme Genes in the Equine Feto-Placental Unit

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The expression of equine cytochrome P450 aromatase gene (CYP19) in various tissues include placenta and fetal gonads were examined by reverse-transcription polymerase chain reaction (RT-PCR), in addition to northern blot analysis of cytochrome P450 steroid 17 α -hydroxylase/C17-20 lyase gene (CYP17) and 3 β -hydroxysteroid dehydrogenase Δ 5- Δ 4 isomerase gene (HSD3B). Prior to this, testicular expression of CYP19 mRNA in adolescence was confirmed by RT-PCR and successive sequencing. The successful amplification of RT-PCR and nucleotide sequence of the PCR products meant the equine testis expresses CYP19 mRNA. In the RT-PCR of the pregnancy samples, CYP19 was expressed only in the placenta and not in the fetal gonads. The equine fetal gonads, which express CYP17, should convert progestins into androgens to supply substrates for aromatization in placenta expressing CYP19. It is suggested that the fetal gonads are the major androgen source in equine gestations, and this function will not be affected by the fetal gender.

Key words: CYP19, CYP17, HSD3B, feto-placental unit

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The research to elucidate the mechanisms of unique hormonal patterns in the equine gestational period is one of the interests in the reproductive biology. A secretion of equine chorionic gonadotropin (eCG) and successive luteinization of accessory corpora lutea and progesterone (P₄) secretion are well-known characteristics in the equine early pregnancy [4, 15]. Furthermore, in the mid-gestational period and thereafter, the fetal gonads become strikingly hypertrophic [12], and in the same time, the maternal blood estrogen levels elevate strikingly [20]. Generally in the mid-gestational period of mammalian species, maternal blood P₄ levels are maintained by the corpora lutea or the placenta. However, in the maternal peripheral P₄ levels are maintained lower for a long time from mid- to late-gestation in horses [19], and P₄ level is lower than the other detectable progestins even in the umbilical venous blood, that flow from placenta to fetus. Several hypotheses presented to explain this phenomenon do not support the insignificance of

progestins in the maintenance of equine gestation. Nevertheless, synthesis of hydroxylated pregnanes, so called "5 α -pregnanes", and emerging increase of their blood levels imply that progestins are actually catabolized in equine placenta [9]. It is estimable to elucidate the relationship and participation of the fetal gonads and placenta in the steroidogenesis during equine gestation period.

Although, relatively high levels of blood estrogens are detected in the gestation period of several mammalian species include humans, their synthetic pathways are not common as well as the significance of their secretion. In addition to estrone (E₁) and estradiol-17 β (E₂), equine specific B-nucleus unsaturated estrogens, equilin and equilenin, their hydroxylates and sulfates are detected from circulating blood and urine samples of pregnant mares [15]. Raeside *et al.* (1979) inferred that the precursors for estrogen synthesis are supplied by fetus because of high levels of dehydroepiandrosterone (DHEA) and pregnenolone (P₅) in the umbilical arterial blood [26]. In this point, synthesis of E₁ and E₂ in the mares during mid- and late- gestation periods might be considered as

the same cooperative basis of fetus and placenta as in the primates [3]. As the possible precursors for equilin and equilenin are exist in the fetal tissues, the participation of fetal gonads to synthesize these equine specific estrogens is also implicated [27].

The final step of estrogen biosynthesis is an aromatization of A-nucleus of the C19 androgens. In the first decades of research in biosynthesis of estrogens, human placenta and equine testis were used [21]. Pigon *et al.* (1961) reported that the amount of estrogens and estrogen derivatives in the urine of mature stallion is higher than the pregnant mares but they decrease after castration [23]. Baggett *et al.* (1959) demonstrated that equine testis can convert testosterone into estrogens [7], and Oh and Tamaoki (1973) corroborated the microsomal localization of aromatization activity in equine testis [22]. Of course, small amount of testicular aromatizations could be observed in many mammalian species, but the extremely high level syntheses of estrogens in equine and porcine testes must be exceptional [32]. Biosyntheses of steroid hormones are regulated by the expression and the function of specific steroidogenic enzymes. 3β -hydroxysteroid dehydrogenase $\Delta 5-\Delta 4$ isomerase (3β -HSD) converts 3β -hydroxy- $\Delta 5$ -steroids into 3-keto- $\Delta 4$ -ene-steroids reversibly, cytochrome P450 steroid 17α -hydroxylase/C17-20 lyase (CYP17) catalyzes 17α -hydroxylation and C₂₀₋₂₁ side chain cleavage, and at last cytochrome P450 aromatase (CYP19) aromatizes androgens into estrogens.

The authors previously reported the northern blot analysis of equine tissues include fetal gonads and placenta with radio labeled cDNA probes of 3β -HSD gene (*HSD3B*), and demonstrated that fetal gonads of both genders do not express *HSD3B* in contrast to placenta, that expresses *HSD3B* in high levels [16]. In this study, we intended to elucidate two points, the expression of *CYP19* in the adult equine testis, fetal gonads and placenta by RT-PCR, and the source and the mechanism of estrogens in equine gestation by multiple tissue northern blot with probes for equine *HSD3B* and *CYP17* in combination with RT-PCR of *CYP19* mRNA.

Materials and Methods

RNA preparations

Tissue samples of adrenal gland, testis, kidney, liver, and gluteus muscle were collected from 2-years-old

Thoroughbred colt, ovaries from 3 days old Thoroughbred foal were collected after necropsy. Corpus luteum (125 days of pregnancy), placentas (125, 180 and 270 days of pregnancy), fetal testis (155 days of pregnancy), and fetal ovary (125 days of pregnancy) were collected by Caesarean sections from pregnant Thoroughbred mares of various ages (from 6 to 15 year old) under sevoflurane-oxygen inhalation anesthesia [1, 16]. The samples were stored in -80°C until extraction of total RNAs. Total RNA was extracted by guanidinium isothiocyanate/CsCl ultracentrifugation [28].

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

First strand cDNAs for RNA samples were synthesized by modified Moroney murine leucopenia virus reverse transcriptase (modified MMLV-RT: SuperScript II preamplification system; GIBCO BRL, Bethesda, U.S.A.). Polymerase chain reactions (PCR) from the first strand cDNAs were performed with GeneAmp PCR System 9600 and GeneAmp PCR Reagent (Perkin Elmer, Urayasu, Japan).

Primers specific to the equine *CYP19* were designed from the nucleotide sequences for equine *CYP19* [2, 11]:

arom-F

5'-GGAATTCGAGAAAGGCATCATATTTAACA-3'

arom-R

5'-GGAATTCCTCAAGAAATCTTAAAGAAGATG-3'

arom-F11

5'-GAATTCGCCTGAGATCAAGGAGCACAAAGATG-3'

arom-F2

5'-GAGAAAGGCATCATATTTAACAATAA-3'

arom-R2

5'-GGAATTCCTCAAGAAATCTTAAAGAAGATGTTTG-3'

The thermal cycle was 95°C for 4 min of pre-denature, and 30 cycles of 95°C for 1 min denature 55°C for 2 min annealing and 72°C for 3 min extension were followed by 72°C for 7 min extension in 100 μl of reaction mixture.

Cloning of the PCR fragment

Molecular size of PCR-amplified fragments were determined by agarose gel electrophoresis (1% agarose in $1 \times \text{TAE}$), and purified with GENE CLEAN II kit (BIO 101, La Jolla, U.S.A.). Purified PCR fragments were ligated into plasmid vector pGEM-T Easy (Promega, Madison, U.S.A.). *Escherichia coli* XL-1 Blue MRF' cells (Stratagene, San Diego, U.S.A.) were

transformed with the ligated DNA. The transformants were selected by blue-white colony selection with IPTG and X-gal on the LB agar plates containing 50 µg/ml ampicillin.

Nucleotide sequencing and computer analysis

Nucleotide sequence of the plasmid inserts were determined by Sanger's dideoxynucleotide chain termination method [29] with the A.L.F. automated sequencer (Pharmacia-Biotech, Tokyo, Japan), fluorescein labeled sequence primers (M4 and RV-M; Takara Shuzo, Kyoto, Japan), and SequiTherm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison, U.S.A.). Obtained sequences were analyzed with the DNA analyzing software package DNASIS-Mac (Hitachi Software Engineering, Yokohama, Japan).

cDNA probes

Inserts were excised from the plasmid clones for equine *CYP17* cDNA [17] by restriction endonuclease *KpnI* and *HSD3B* cDNA [16] by restriction endonuclease *BamHI*. Excised inserts were purified with GeneClean II kit (BIO 101, La Jolla, U.S.A.), and labeled with [α -³²P] dCTP (Rediprime DNA Labeling System; Amersham, Tokyo, Japan).

Northern blot analysis

Multiple tissue blots were performed with RNA samples described above. Twenty micrograms of total RNA from each tissue was separated by electrophoresis in 1.0% agarose 3-[N-morpholino]-propanesulfonic acid (MOPS)-formamide-formaldehyde gel [28]. Separated nucleic acids were transferred to a piece of positively charged nylon membrane (HyBond N⁺; Amersham, Tokyo, Japan), and hybridized with the radio labeled probe for *HSD3B*. After the detection of *HSD3B*, probe for *HSD3B* was stripped by boiling in 50% formamide 6 × SSPE for 30 min at 65°C and reprobed with the radio labeled probe for *CYP17*. BAS imaging plate scanner (BAS2500 system; Fuji Photo Film, Tokyo, Japan) was used to visualize the localization of radioactivities.

Results

The results of agarose gel electrophoresis of the RT-PCR products were shown in Fig. 1. PCR products around 350 bp from a pair of primers arom-F2/arom-R and around 750 bp from another pair of primers arom-

F11/arom-R2 were detected in samples from testis of an adult (adolescent) colt, and placentas (days 125, 180 and 270 of pregnancy). There were no signs of amplification in the samples from adrenal gland, corpus luteum (day 125 of pregnancy), fetal ovary (day 125 of pregnancy), fetal testis (day 155 of pregnancy), and 3-days-old foal ovary, as well as adult non-steroidogenic liver, kidney and muscle. Sequence analysis was performed on the cloned PCR products from testicular cDNA. The exact length of the shorter PCR fragment around 350 bp was 351 bp, and that for the longer one was 763 bp. The nucleotide sequences for both two kinds of PCR fragments were identical to the previously reported *CYP19* sequences (Fig. 2) [2, 8]. The shorter product was completely included in the longer one, and the nucleotide sequences of the redundant part were identical with each other, as well as with the reported sequence.

By northern blot analysis, the equine *CYP17* mRNAs were expressed in adult testis, adrenal gland and fetal gonads (Fig. 3), in contrast to the equine *HSD3B* mRNAs, that expressed in adult testis, adrenal gland, corpus luteum, and placenta, but not detected in fetal gonads of both genders [22]. Non-steroidogenic tissues, liver, kidney and muscle did not express both two steroidogenic enzymes, while the 3-days-old foal ovary expressed both. The expression levels of *CYP17* in the fetal gonads were higher than the testis and the adrenal gland.

Discussion

Metabolic pathways of steroid synthesis are so common in various species that the similarity of function or structure of steroidogenic enzyme in each step is conserved over species. In steroidogenic tissues, species of steroid hormone as the final products are determined by kinds of substrates, natures and expression levels of steroidogenic enzymes. The authors previously reported on the cDNA cloning of *CYP17* [17] and *HSD3B* [16]. It is obvious that estrogens are synthesized in the equine testis by old literature [7], and *CYP19* seemed to be expressed in the equine testis. Actually, early studies of conversion from androgens to estrogens had been conducted using equine testis [21] but it is more recent that equine *CYP19* has been demonstrated as a single protein molecule in an equine testis [5, 10, 14, 30].

As result of RT-PCR experiment, a 351 bp (primers

Fig. 1. Agarose gel electrophoresis of RT-PCR products. A: RT-PCR products for primers arom-F11/R2, B: RT-PCR products for primers arom-F2/R, M: molecular size marker (50–1000 bp), 1: adrenal gland*, 2: testis*, 3: corpus luteum (125 dpc), 4: placenta (125 dpc), 5: placenta (180 dpc), 6: placenta (270 dpc), 7: fetal ovary (125 dpc), 8: fetal testis (155 dpc), 9: ovary (3 dpd), 10: liver*, 11: kidney*, 12: muscle*, dpc: days post coitus, dpd: days post delivery, *: 2 to 3 years old adults.

arom-F2/arom-R2) and a 763 bp (primers arom-F11/arom-R) PCR products were amplified from equine testicular RNA sample (Fig. 1). We confirmed that the nucleotide sequences of the PCR products were identical to the previously reported equine *CYP19* cDNA expressed in a primary corpus luteum at early pregnancy [2] and human chorionic gonadotropin (hCG) treated ovarian follicles [8]. The nucleotide sequence of the shorter 351 bp PCR product was completely included in those of the longer 763 bp product.

Other than the adult testis, two kinds of PCR products were only detected in 3 samples of placentas (Fig. 1). It is in concomitant with previous report on the placental localization of *CYP19* protein [18]. The failure to amplification by both two sets of primers from other tissues implies that *CYP19* mRNA was not or rarely expressed in these tissues. The corpus luteum is known to express *CYP19* in the early pregnancy. Albrecht *et al.* (1997) demonstrated that the corpora lutea of cycling mares, days from 29 to 35 and from 42 to 45 of pregnancy express *CYP19* mRNA. They

discussed that the luteal expression of *HSD3B*, *CYP17* and *CYP19* is constant before the onset of eCG secretion, but promotional effect of eCG on the *CYP17* expression results in the increase of estrogen production [2]. In this study, the corpus luteum at mid-gestation showed no evidence for expression of *CYP17* and *CYP19*, so the luteal contribution to the synthesis of androgens and estrogens seemed to be very limited in this period. Furthermore, as the luteal expression of *HSD3B* in this period is lower than that of placenta in same period, it is suggested that corpus luteum handed over the responsibility supplying C21 steroid substrates necessary to maintain pregnancy.

In the ovarian follicles, granulosa cells and thecal cells cooperate with each other to synthesize estrogens, and in this system, so called “Armstrong-Dorrington’s two-cell model” [6], granulosa cells express *HSD3B* and *CYP19* while thecal cells express *CYP17*. These concepts were also accepted to be applicable in the fetoplacental unit [3]. In the human fetoplacental unit, the estrogen synthesis in late-gestation is supported by the cooperative function of the placenta

		10	20	30	40	50	
AF031520	1	TTCTTAACAG	CCGTGCATCA	TTAGCAAAAC	TCATCATCTT	CAAGAGTCCG	50
AF031520	51	GAAACTAGAA	GTGACCAGCA	GACTCAGGCC	TTTACATTGC	TTGCGCTGAG	100
aromF11/R2	1				GAA	TTGCGCTGAG	13
AF031520	101	ATCAAGGAAC	ACAAGATGAT	TTTGAAATG	CTAAACCCGA	TGCATTATAA	150
aromF11/R2	14	ATCAAGGAGC	ACAAGATGAT	TTTGAAATG	CTAAACCCGA	TGCATTATAA	63
AF031520	151	CCTCACCAGC	ATGGTGCCCG	AAGTCATGCC	TGTCGCCACC	TTGCCCATTC	200
aromF11/R2	64	CCTCACCAGC	ATGGTGCCCG	AAGTCATGCC	TGTCGCCACC	TTGCCCATTC	113
AF031520	201	TGCTGCTCAC	TGGCTTTCTT	TTCTTTGTTT	GGAATCATGA	AGAAACATCC	250
aromF11/R2	114	TGCTGCTCAC	TGGCTTTCTT	TTCTTTGTTT	GGAATCATGA	AGAAACATCC	163
AF031520	251	TCAATACCAG	GCCCTGGCTA	TTGCATGGGA	ATCGGGCCCC	TCATTTCCTA	300
aromF11/R2	164	TCAATACCAG	GCCCTGGCTA	TTGCATGGGA	ATCGGGCCCC	TCATTTCCTA	213
AF031520	301	CCTCCGGTTC	CTGTGGATGG	GGCTTGGCAG	TGCCTGCAAC	TACTACAACA	350
aromF11/R2	214	CCTCCGGTTC	CTGTGGATGG	GGCTTGGCAG	TGCCTGCAAC	TACTACAACA	263
AF031520	351	AGATGTATGG	AGAATTCGTA	AGAGTCTGGA	TCAGTGGAGA	GAAAACGCTC	400
aromF11/R2	264	AGATGTATGG	AGAATTCGTA	AGAGTCTGGA	TCAGTGGAGA	GAAAACGCTC	313
AF031520	401	GTTATTAGCA	AGTCCCTCAAG	TACCTTCCAC	ATCATGAAAC	ACGATCACTA	450
aromF11/R2	314	GTTATTAGCA	AGTCCCTCAAG	TACCTTCCAC	ATCATGAAAC	ACGATCACTA	363
AF031520	451	CTCCTCCCGA	TTTGGCAGCA	CATTTGGGTT	GCAGTATATG	GGCATGCATG	500
aromF11/R2	364	CTCCTCCCGA	TTTGGCAGCA	CATTTGGGTT	GCAGTATATG	GGCATGCATG	413
aromF2/R	1					G	1
AF031520	501	AGAATGGCGT	CATATTTAAC	AATAACCCAG	CCGTCTGGAA	AGCTTTGCGG	550
aromF11/R2	414	AGAATGGCGT	CATATTTAAC	AATAACCCAG	CCGTCTGGAA	AGCTTTGCGG	463
aromF2/R	2	AGAATGGCGT	CATATTTAAC	AATAACCCAG	CCGTCTGGAA	AGCTTTGCGG	51
AF031520	551	CCTTTCTTTG	TAAAAGCTTT	GTCTGGCCCC	AGCCTTGCGC	GCATGGTGAC	600
aromF11/R2	464	CCTTTCTTTG	TAAAAGCTTT	GTCTGGCCCC	AGCCTTGCGC	GCATGGTGAC	513
aromF2/R	52	CCTTTCTTTG	TAAAAGCTTT	GTCTGGCCCC	AGCCTTGCGC	GCATGGTGAC	101
AF031520	601	AGTTTGTGTT	GAATCCGTCA	ACAACCATCT	GGACAGGTTG	GACGAGGTCA	650
aromF11/R2	514	AGTTTGTGTT	GAATCCGTCA	ACAACCATCT	GGACAGGTTG	GACGAGGTCA	563
aromF2/R	102	AGTTTGTGTT	GAATCCGTCA	ACAACCATCT	GGACAGGTTG	GACGAGGTCA	151
AF031520	651	CCAATGCGTT	GGGCCATGTC	AACGTGTTGA	CCCTCATGCG	ACGTACCATG	700
aromF11/R2	564	CCAATGCGTT	GGGCCATGTC	AACGTGTTGA	CCCTCATGCG	ACGTACCATG	613
aromF2/R	152	CCAATGCGTT	GGGCCATGTC	AACGTGTTGA	CCCTCATGCG	ACGTACCATG	201
AF031520	701	CTGGACGCTT	CCAACACCCCT	CTTCCTGAGG	ATCCCCTTGG	ACGAGAAAAA	750
aromF11/R2	614	CTGGACGCTT	CCAACACCCCT	CTTCCTGAGG	ATCCCCTTGG	ACGAGAAAAA	663
aromF2/R	202	CTGGACGCTT	CCAACACCCCT	CTTCCTGAGG	ATCCCCTTGG	ACGAGAAAAA	251
AF031520	751	CATCGTGCTT	AAAATCCAGG	GTTATTTTGA	TGCATGGCAG	GCTCTCCTTA	800
aromF11/R2	664	CATCGTGCTT	AAAATCCAGG	GTTATTTTGA	TGCATGGCAG	GCTCTCCTTA	713
aromF2/R	252	CATCGTGCTT	AAAATCCAGG	GTTATTTTGA	TGCATGGCAG	GCTCTCCTTA	301
AF031520	801	TCAAACCAAA	CATCTTCTTT	AAGATTTCCT	GGCTATCCAG	AAAGCATCAA	850
aromF11/R2	714	TCAAACCAAA	CATCTTCTTT	AAGATTTCCT	GGAATTCC...		763
aromF2/R	302	TCAAACCAAA	CATCTTCTTT	AAGATTTCCT	GGAATTCC...		351

Fig. 2. Nucleotide sequence of RT-PCR products for equine *CYP19*. PCR primers were indicated with box shadows. AF031520: complete cDNA sequence for equine *CYP19* [8], arom-F11/R2 and arom-F2/R: see text.

expressing β -HSD and CYP19, and the fetal adrenal gland expressing CYP17. As the adrenal gland of term human fetus has also β -HSD activities, the fetal adrenal gland may possible to produce androgens from

P₅ or cholesterol [13]. In the adrenal cortex, distinct populations of steroidogenic enzymes are expressed. The each zone of the adrenal cortex secretes distinct products, aldosterone from zona glomerulosa, cortisol

Fig. 3. Multiple tissue northern blot analysis for *HSD3B* and *CYP17*. 20 μ g of total RNA in each lane was analyzed. Adult adrenal gland, testis, and non-steroidogenic control, liver, kidney and muscle, were also analyzed. dpc: days post coitus, dpd: days post delivery.

or corticosterone from zona fasciculata, and zona reticularis sulfonates unnecessary DHEA by sulfokinase. DHEA sulfonate (DHEA-S) produced by human fetal adrenocortex may be catalyzed to DHEA by placental sulfatase and aromatized in placenta. Thus, in primates, fetal adrenal gland is an additional major source of DHEA as a substrate to produce estrogens as well as maternal adrenal gland.

In horses, B-unsaturated equine estrogens, equilin and equilenin, E_1 , estradiol- 17β and - 17α exist in the maternal blood and urine at high levels. Although placenta seemed responsible to produce estrogens, as estrogens could be synthesized by incubating placental tissue with various precursors. Raeside *et al.* (1973) inferred that equine fetal gonads are responsible to the placental estrogen synthesis as well as human fetal adrenal because of drastic decrease of estrogen levels in maternal urine after removal of fetal gonads [25]. Here we compared the expression of genes for major steroidogenic enzymes, *HSD3B*, *CYP17* and *CYP19* in the fetal gonads and placenta, and the prominent expression of *CYP17* in the fetal gonads was observed while the placenta showed no expression. In contrast to *CYP17*, *HSD3B* and *CYP19* was expressed in the placenta, while the fetal gonads showed no expression.

The observations suggested that the estrogen synthesis in the equine pregnancy is supported by a cooperative function to supply substrates between fetus and placenta. Since there are high levels of P_5 and

DHEA in equine fetal gonads and gonadal venous blood [24], equine fetal gonads may produce DHEA from P_5 as a substrate by *CYP17*. $\Delta 4$ -steroids may not be produced by fetal gonads because of lack of *HSD3B* expression, and DHEA may be a final product in fetal gonads. Therefore, it is suggested that $\Delta 4$ -androgens necessary for estrogen synthesis by placental *CYP19* are converted from DHEA, supplied by fetus, by placental 3β -HSD. While blood testosterone levels in pregnant mares vary like estrogens [31], this testosterone should be synthesized by placenta because of lack of *HSD3B* expression in fetal gonads. In this study, fetal adrenal gland had not been tested. It may be important to discuss the contribution of fetal adrenal gland to the fetoplacental steroid synthesis by DHEA-S pathway, but linear growth of the equine fetal adrenal gland in mid-gestation to parturition [33] is different from the patterns in the equine fetal gonads, that grow quickly and shrink by late-gestation [4, 12, 15]. The results obviously showed the importance of fetal gonads at least the source of androgen substrate for production of maternal blood estrogens.

Compared with humans, the mechanism of estrogen synthesis in the equine fetoplacental unit might be essentially identical only exception that the major contributing organs are gonads, while adrenal gland in humans. However, there may be differences between two species about the significance of increase in maternal estrogen levels. In humans, maternal

estrogen levels increase continuously, and this may induce various function related to parturition. In ruminants, increase of cortisol in late-gestational period induces placental *CYP17*, and resultant increase of estrogen level promotes parturition. In horses, it is not acceptable that high levels of estrogens is necessary to induce parturition in human alike, because maternal blood levels of estrogens decrease with the size of fetal gonads, and the decrease of estrogens occurs very early in a basis of parturition timing without rebound just before parturition. Estrogens, which increase in mid-to late-pregnancy, may act on uterus or uterine blood supply in cooperation with progestins, and inhibit secretion of gonadotropins to maintain pregnancy.

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References

1. Aida, H., Mizuno, Y., Hobo, S., Yoshida, K., and Fujinaga, T. 1996. Cardiovascular and pulmonary effects of sevoflurane anesthesia in horses. *Vet. Surg.* **25**: 164–170.
2. Albrecht, B.A., MacLeod, J.N., and Daels, P.F. 1997. Differential transcription of steroidogenic enzymes in the equine primary corpus luteum during diestrus and early pregnancy. *Biol. Reprod.* **56**: 821–829.
3. Albrecht, E.D. and Pepe, G.J. 1990. Placental steroid hormone biosynthesis in primate pregnancy. *Endocr. Rev.* **11**: 124–150.
4. Allen, W.R. 2001. Fetomaternal interactions and influences during equine pregnancy. *Reproduction* **121**: 513–527.
5. Almadhidi, J., Seralini, G.E., Fresnel, J., Silberzahn, P., and Gaillard, J.L. 1995. Immunohistochemical localization of cytochrome P450 aromatase in equine gonads. *J. Histochem. Cytochem.* **43**: 571–577.
6. Armstrong, D.T. and Dorrington, J.H. 1977. Estrogen biosynthesis in the ovaries and testes. *Adv. Sex Horm. Res.* **3**: 217–258.
7. Baggett, B., Engel, L.L., Balderas, L., Lanman, G., Savard, K., and Dorfman, R.I. 1959. Conversion of C14-Testosterone to C14-estrogenic steroids by endocrine tissues. *Endocrinol.* **64**: 600–608.
8. Boerboom, D., Kerban, A., and Sirois, J. 1999. Dual regulation of promoter II- and promoter I-derived cytochrome P450 aromatase transcripts in equine granulosa cells during human chorionic gonadotropin-induced ovulation: a novel model for the study of aromatase promoter switching. *Endocrinol.* **140**: 4133–4141.
9. Chavatte, P., Holtan, D., Ousey, J.C., and Rossdale, P.D. 1997. Biosynthesis and possible biological roles of progestagens during equine pregnancy and in the newborn foal. *Equine Vet. J., Suppl.* **24**: 89–95.
10. Choi, I., Collante, W.R., Simmen, R.C.M., and Simmen, F.A. 1997. A developmental switch in expression from blastocyst to endometrial/placental-type cytochrome P450 aromatase genes in the pig and horse. *Biol. Reprod.* **56**: 688–696.
11. Choi, I., Simmen, R.C., and Simmen, F.A. 1996. Molecular cloning of cytochrome P450 aromatase complementary deoxyribonucleic acid from periimplantation porcine and equine blastocysts identifies multiple novel 5'-untranslated exons expressed in embryos, endometrium, and placenta. *Endocrinol.* **137**: 1457–1467.
12. Cole, H.H., Hart, G.H., Lyons, W.R., and Catchpole, H.R. 1933. The development and hormonal content of fetal horse gonads. *Anat. Rec.* **56**: 275–293.
13. Coulter, C.L., Goldsmith, P.C., Mesiano, S., Voytek, C.C., Martin, M.C., Mason, J.I., and Jaffe, R.B. 1996. Functional maturation of the primate fetal adrenal *in vivo*. II. Ontogeny of corticosteroid synthesis is dependent upon specific zonal expression of 3 β -hydroxysteroid dehydrogenase/isomerase. *Endocrinol.* **137**: 4953–4959.
14. Eisenhauer, K.M., McCue, P.M., Nayden, D.K., Osawa, Y., and Roser, J.F. 1994. Localization of aromatase in equine Leydig cells. *Domest. Anim. Endocrinol.* **11**: 291–298.
15. Ginther, O.J. 1992. *In: Reproductive Biology of the Mare—Basic and Applied Aspects—2nd ed.*, 1992, Cross Plains: Equiservices.
16. Hasegawa, T., Ishida, M., Harigaya, T., Ishida, N.,

- and Mukoyama, H. 1998. Molecular cloning, nucleotide sequence and tissue distribution of equine testicular 3β -hydroxysteroid dehydrogenase/ $\Delta 5$ - $\Delta 4$ isomerase messenger ribonucleic acid. *J. Equine Sci.* **9**: 45–52.
17. Hasegawa, T., Mukoyama, H., Yoshida, S., and Takahashi, M. 1995. Molecular cloning and nucleotide sequence of equine testicular cytochrome P450 steroid 17α -hydroxylase/ $C17,20$ -lyase messenger ribonucleic acid. *Biol. Reprod. Mono.* **1**: 615–622.
 18. Hinshelwood, M.M., Liu, Z., Conley, A.J., and Simpson, E.R. 1995. Demonstration of tissue-specific promoters in nonprimate species that express aromatase P450 in placenta. *Biol. Reprod.* **53**: 1151–1159.
 19. Holtan, D.W., Houghton, E., Silver, M., Fowden, A.L., Ousey, J., and Rosedale, P.D. 1991. Plasma progestagens in the mare fetus and newborn foal. *J. Reprod. Fertil., Suppl.* **44**: 517–528.
 20. Nett, T.M., Holtan, D.W., and Estergreen, V.L. 1973. Plasma estrogens in pregnant and postpartum mares. *J. Anim. Sci.* **37**: 962–970.
 21. Oh, R. and Tamaoki, B.-i. 1971. Occurrence of 19-oxoandrost-4-ene-3,17-dione in the course of oestrogen biosynthesis by equine testicular microsomes. *Acta Endocrinol. (Copenh)* **67**: 665–676.
 22. Oh, R. and Tamaoki, B.-i. 1973. Intermicrosomal distribution of aromatizing enzyme system in equine testicular tissue. *Acta Endocrinol. (Copenh)* **72**: 366–375.
 23. Pigon, H., Lunaas, T., and Velle, W. 1961. Urinary oestrogens in the stallion. *Acta Endocrinol.* **36**: 131–140.
 24. Raeside, J.I., Gofton, N., Liptop, R.M., and Milne, F.J. 1982. Isolation and identification of steroids from gonadal vein blood of the fetal horse. *J. Reprod. Fertil., Suppl.* **32**: 383–387.
 25. Raeside, J.I., Liptrap, R.M., and Milne, F.J. 1973. Relationship of fetal gonads to urinary estrogen excretion by the pregnant mare. *Am. J. Vet. Res.* **34**: 843–845.
 26. Raeside, J.I., Liptrap, R.M., and Milne, F.J. 1979. A precursor role for DHA in a fetoplacental unit for oestrogen formation in the mare. *J. Reprod. Fertil., Suppl.* **27**: 493–497.
 27. Raeside, J.I. and Renaud, R.L. 1985. Identification of 3β -hydroxy-5,7-androstadien-17-one as a secretory product of the fetal horse gonad *in vivo* and *in vitro*. *J. Endocrinol.* **107**: 415–419.
 28. Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *In: Molecular Cloning: A Laboratory Manual* 2 ed. 1989, Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
 29. Sanger, F., Nicklen, S., and Coulson, A.R. 1989. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74**: 5463–5467.
 30. Silberzahn, P., Gaillard, J.L., Quincey, D., Dintinger, T., and Al-Timimi, I. 1988. Aromatization of testosterone and 19-nortestosterone by a single enzyme from equine testicular microsomes. Differences from human placental aromatase. *J. Steroid Biochem.* **29**: 119–125.
 31. Silberzahn, P., Zwain, I., and Martin, B. 1984. Concentration increase of unbound testosterone in plasma of the mare throughout pregnancy. *Endocrinol.* **115**: 416–419.
 32. Velle, W. 1966. Urinary oestrogens in the male. *J. Reprod. Fertil.* **12**: 65–73.
 33. Yamauchi, S. 1979. Histological development of the equine fetal adrenal gland. *J. Reprod. Fertil., Suppl.* **27**: 487–491.