Changes of testicular aromatase expression during fetal development in male pigs (*sus scrofa*)

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Abstract

Male pig fetuses secrete considerable amounts of estrogens, but the location of aromatase activity within the fetal testis is not known. The location of aromatase expression was investigated by immunocytochemistry in fetal testes from week 6 (*n* = 5), weeks 10, 13, and 15 (each: *n* = 6) of gestation and additionally in neonates (*n* = 4). Blood was sampled from the umbilical artery of fetuses and jugular vein of neonates. Histological evaluation of testes involved morphological criteria and counting of Leydig cells, Sertoli cells, and gonocytes. Aromatase activity was localized immunocytochemically and quantified by the percentage of positive stained cells within the same cell type. Aromatase expression was further characterized by quantitative RT-PCR. Concentrations of estrogens, testosterone, FSH, and LH were measured in blood plasma. Total estrogens increased from week 10 to a maximum of 31.03 nmol/l in week 15. Increased testosterone concentrations were only measured at week 6 and were paralleled by slightly elevated estrogens. Thereafter, testosterone dropped and was low throughout. The increase of estrogens was not paralleled by a similar increase of FSH and LH but was related to the increase of the total number of Leydig cells. This increase was also found for mRNA expression. Both Leydig cells and gonocytes were identified as contributors to estrogen formation. Gonocytes were the main source of aromatase at week 10, when gene expression by Leydig cells is low due to the preparation of a wave of Leydig cell mitosis.


Introduction

Formation of androgens during embryonic and fetal development of males was investigated in many species due to their role for sexual differentiation (e.g., Attal 1969, Corpechot et al. 1981, Rommerts et al. 1982). In addition, testicular biosynthesis of low amounts of estrogens was reported during fetal life. It was characterized by determination of aromatase enzyme activity in testicular tissue homogenates which was found to increase along fetal development (Tsai-Morris et al. 1985, Greco & Payne 1994). Immunocytochemical localization of aromatase in the testes during fetal development was not performed.

In pigs a peculiar situation exists, because sexually mature males not only synthesize androgens but also high amounts of estrogens. Blood plasma concentrations in boars even exceed those which are known to occur in estrous females (Claus & Hoffmann 1980, Claus et al. 1987). Together with androgens, they play a synergistic role to support accessory sex gland function, male growth characteristics, and behavior (Booth 1988, Lewis & Ford 1989). In consequence, their formation is highly correlated with androgens and occurs exclusively in Leydig cells of mature boars (Raeside et al. 1993). Steroid formation in testes of pig fetuses revealed a first rise of androgen biosynthesis around 30 days postcoitum (Ford et al. 1980). Mesenchymal Leydig cells were identified as the primary source of testosterone at that stage of development (Pelliniemi 1975, Pelliniemi et al. 1979).

The following rapid maturation of Leydig cells is reflected by a testosterone maximum between 35 and 38 days. Thereafter, testosterone concentrations decrease and remain low during further fetal development (Ford et al. 1980). Another considerable rise was not demonstrated before an age of 4–6 weeks postnatally (Schwarzenberger et al. 1993). The trophoblast is the first source of estrogens in both male and female embryos. These estrogens inhibit prostaglandin F2α formation and thus luteolysis (e.g., Flint et al. 1978).

Measurements of estrogens in testis homogenates led to the conclusion that estrogen formation also occurs during fetal development (Raeside et al. 1993). Determination of estrogens in plasma of the umbilical artery revealed that concentrations are similar for male and female fetal pigs (Ford & Christenson 1986).
In newborn piglets, aromatase activity was found to occur only in Leydig cells (Conley et al. 1996). The localization of the aromatase complex by immunocytochemistry was not performed in fetuses so far.

Materials and Methods

All steps of the animal experiments, including cannulation of sows, sampling of blood, fetus collection, and killing were approved by the local animal welfare commission.

Characterization of sows

For the experiment, four groups of German multiparous landrace sows were included. Each group was represented by four sows. All sows of a group had been mated with Pietrain boars within the same week. They were killed for fetus collection at either 6, 10, 13, or 15 weeks of pregnancy.

The sows were kept conventionally in stalls of the experimental farm of the University Hohenheim. They were fed a standard diet for pregnant sows with 12.7 MJ metabolizable energy/kg feed and 11.8% crude protein. Sows at an early stage of gestation (<85 days) were fed 2.2 kg/day, and at an advanced stage of gestation (>85 days) were fed 2.8 kg/day. Four weeks before fetus collection, each group of sows was transferred to the experimental unit. After 2 weeks of adaptation to the new environment, they were fitted with indwelling cephalic vein catheters for blood collection as described previously (Claus et al. 1990). After 1 week recovery, blood samples were taken twice daily at 8 and 15 h over the following week before killing and removal of fetuses.

Fetus collection and sampling

Tissue and blood samples were taken from fetuses of weeks 6, 10, 13, and 15 of gestation and additionally from piglets the day of birth. For fetus collection, sows were anesthetized by i.m. injection of Stresnil (Janssen-Cilag, Neuss, Germany) at an amount of 10 mg/kg body weight, and ketamin (Ursotamin, Serumwerk Bamberg Cilag, Neuss, Germany) at an amount of 2 mg/kg body weight. Anesthesia was maintained by a mixture of O₂/N₂O (ratio 2:1.5) and isofluran (Baxter, Unterscheiβheim, Germany) at an amount of 10 mg/kg body weight. After an incision along the linea alba, the uterus horns were exteriorized. Position of the fetuses was palpated, the uterus opened for individual removal of fetuses. Blood samples were aspirated after puncture of the umbilical artery. Heparinized plasma was stored at −20°C for hormone analysis. At week 6, sufficient volumes of plasma could only be sampled from a few fetuses.

Thereafter, the fetuses were separated from their mothers and killed by injection of 0.3 ml/kg body weight T61 (Intervet Deutschland GmbH, Unterscheiβheim, Germany). Altogether 78 male and 80 female fetuses were collected. For this paper, however, testes and blood (umbilical artery) from six male fetuses were evaluated for each gestation stage, except week 6, where only five male fetuses were available. The testes were removed within 10 min postmortem. One testis from each fetus was fixed in 4% formaldehyde for histological evaluation, the other one in RNA later for RNA extraction and mRNA quantification. RNA later solution composed of 70% ammonium sulfate, 0.5 M EDTA, 1 M sodium citrate, and diethylypyrocarbonate water. The length and the diameter of the testes were determined and the volume was calculated.

Sampling of newborn piglets

In addition to fetuses, testes, and jugular vein blood plasma were also sampled by venipuncture from four newborn piglets (6 and 12 h after birth) for hormone analysis and histological and molecular evaluation respectively. These piglets were also German Landrace × Pietrain crossbreds, and obtained from the university herd. They were killed by injection of 0.2 ml Eutha 77 per kg body weight (Essex Pharma, Munich, Germany) into the carotic artery and all samples could again be obtained within 10 min after killing.

Hormone analysis

The following hormones were measured by specific RIAs in umbilical artery plasma and peripheral plasma of neonates: luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and estrogens. Estrogens were additionally determined in peripheral plasma of sows. Steroids were determined after solvent extraction, so that the values represent unconjugated steroids. The details of the RIAs had been reported previously and are only described in brief below.

Determinations of LH and FSH were performed by RIA as published earlier (Claus et al. 1990), with the exception of the first antibody being preincubated with the sample before adding the tracer in the case of LH. For iodination LH (AFP: 11043B) and FSH (AFP: 10640B) were obtained from Dr Parlow (NIDDK, Torrance, CA, USA). From the same source, the species-specific antisera (LH: AFP 15103194; FSH: AFP 2062096Rb) were obtained. The LH antiserum could be used at a final dilution in the ratio of 1:660 000 and the FSH antiserum at a dilution in the ratio of 1:200 000.

The reliability criteria were as follows. For LH, the sensitivity was 0.83 pmol/l blood plasma. The intra-assay coefficient of variation was 7.9% and the interassay coefficient was 8%. And for FSH, the sensitivity was
1.20 pmol/l blood plasma. The intra-assay coefficient of variation was 11% and the interassay coefficient was 12%.

The assay for testosterone (Bubenik et al. 1982) was based on an antiserum against testosterone-3-carboxymethyl-oxime-bovine serum albumin (testosterone-3-CMO-BSA) and revealed cross-reactivity only with 5α-dihydrotestosterone (28.5%). It was used at a dilution in the ratio of 1:70 000. The sensitivity of the assay was 0.14 nmol/l plasma. The intra-assay coefficient of variation varied between 3.9 and 6.7% depending on concentration and for the interassay coefficient of variation varied between 7 and 18%. Total estrogens (Claus et al. 1985) were determined with an antiserum which had been raised against estrone-17-CMO-BSA. It was used at a dilution in the ratio of 1:15 000. Extraction was performed with 3 ml tertiary butyl-methyl ether. The sensitivity was 73.5 pmol/l plasma, the intra-assay coefficient of variation varied between 2.9 and 3.7% and the interassay coefficient of variation varied between 10.7 and 16%. In addition to total estrogens, the concentrations of 17β-estradiol were determined in two randomly selected male fetuses from weeks 10, 13, and 15 (Claus et al. 1985). The antiserum had been raised against 17β-estradiol-6-CMO-BSA. This antiserum revealed cross-reactivities only with 17α-estradiol (0.4%) and estrone (0.43%). The sensitivity was 42.3 pmol/l. The intra-assay coefficient of variation was 18% for a concentration of 316.1 pmol/l. The interassay variation varied depending on the concentration between 10 and 16%.

**Histological techniques**

The formaldehyde fixed samples were conventionally dehydrated and embedded in paraffin (Romeis 1989). Sections of 4 μm were cut with a Leica sliding microtome (SM 2000R, Nussbach, Germany).

The determination of aromatase was based on a polyclonal antibody raised in rabbits against human aromatase (gift from Dr N Harada; Fujita Health University, Toyoake, Japan). It was used at a dilution in the ratio of 1:1000 and was previously shown to be also specific in the pig (Moran et al. 2002, Weng et al. 2005).

Sertoli cells were determined with the aid of the Sertoli cell marker GATA-4 as described previously by McCoard et al. (2001). GATA-4 was localized immunocytochemically with a commercial purified goat anti-mouse polyclonal antiserum (C-20: Santa Cruz Biotechnology, Santa Cruz, CA, USA). It is exclusively expressed by Sertoli cells of the testis (McCoard et al. 2001). Both for aromatase and GATA-4 staining sections were deparaffinized and rehydrated through graded ethanol, microwaved five times (800 W) for 5 min in 0.01 M citrate buffer (pH 6.0) and treated with 3% H2O2 to quench endogenous peroxidase activity. Sections were then incubated with normal sheep serum (aromatase; 1:10) and rabbit serum (GATA-4; 1:10) respectively, to block unspecific binding. After incubation for an hour with the relevant antibodies, the sections were washed and incubated with the corresponding biotinylated secondary antibody for 30 min (aromatase: sheep anti-rabbit 1:400; Dako Cytomation, Glostrup, Denmark; GATA-4: rabbit anti-goat 1:400; Biozol, Eching, Germany). Immunodetection was then visualized using the avidin–biotin-peroxidase system (Dako Cytomation) with diaminobenzidine as a chromogen. Testis tissue was additionally incubated without the primary antibodies to exclude unspecific binding of the secondary antibody.

To test the specificity of the primary antibody, tissue sections were incubated with preimmune serum from goat (GATA-4). Dilutions of rabbit-IgG were used for testing specificity of the primary antibody against aromatase. Comparative incubations with first antibody or preimmune serum and IgG respectively were performed on the same slide (see Fig. 2E and F).

Counterstaining was performed by hematoxylin. Testis tissue from mature boars served as internal control.

**Histological evaluation**

The diameters of 100 round tubule cross-sections were determined at a magnification of 400× and tested for significant differences. Since the coefficient of variation of tubular diameters was 16% counts for the remaining animals were restricted to ten tubules per animal.

Sertoli cell numbers were determined on the basis of GATA-4 staining. Stained cells from ten round tubules were counted and are given as the mean number per tubule. The number was corrected for nuclear size and section thickness by the method of Abercrombie (1946). Therefore, the nuclear diameter of Sertoli cells was obtained as the mean of its larger and smaller diameters.

Gonocytes were counted in ten round tubules from each animal. Similar to Sertoli cells, they were corrected for nuclear diameter. The total number of Leydig cells was determined with a grid on ten randomly selected areas per slide and referred to a total area of 1 mm². The nuclei were also corrected for section thickness and size.

Aromatase positive Leydig cells were counted with a grid within a total area of 1 mm² (average of ten areas per slide). The counted cell number is given as ‘positive Leydig cells per millimeter square’. In addition, aromatase positive gonocytes were counted in ten tubules per slide and the number is given as positive gonocytes per tubule. These counts were also corrected. Because of their big, round shape the gonocytes could be clearly distinguished from the smaller and ovoid Sertoli cells.

**mRNA determination**

Aromatase expression was additionally determined by quantitative real-time RT-PCR in all testes with the
exception of the 6-week-old fetuses where the size of the gonads was too small for RT-PCR.

**RNA preparation**

First, total RNA was prepared and purified from fetal testes tissue (20–30 mg) using Nucleospin RNA II (Machery and Nagel; Düren, Germany), as described previously. The quantity and quality of the extracted RNA was determined spectroscopically through u.v. measurement at an absorbance of 260 and 260/280 nm respectively, using a Biophotometer (Eppendorf; Hamburg, Germany). RNA was considered to be of good quality if the ratio 260:280 nm was between 1.7 and 2. Otherwise new RNA from fresh tissue was extracted. Additionally, the quality of RNA was assessed by the presence of 28S and 18S bands after RNA gel electrophoresis.

**RT and PCR (RT-PCR)**

First-strand cDNA synthesis was carried out in a 50 μl reaction mixture containing 1 μg total RNA, 2.5 μM random hexamers (Invitrogen) and M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase RNase H minus mutant (200 μl⁻¹; Promega) according to Gabler et al. (1997). A negative RT-reaction (RT-enzyme was replaced by water) was performed to detect residual DNA contamination. The following primers were synthesized commercially (MWG, Ebersberg, Germany):

- GAPDH (Glyceraldehyde-3-phosphate-dehydrogenase) as a housekeeping gene (multipurpose primer; Berisha et al. 2002): 197 bp forward (5'-3') GTC ACT ACC ATG GAG AAG G, reverse (5'-3') TCA TGG ATG ACC TTG GCC AG.
- Aromatase (pig-specific SSU92246; 190 bp) forward (5'-3') CTC GAG TTT TTC CCC AAG C, reverse (5'-3') ACT GGC CTT GCT GTG TTT G.

The annealing temperature of each primer pair was first optimized in a gradient thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). Subsequent RT-PCR was performed with 1.5 μl cDNA (30 ng reverse transcribed total RNA) at a final volume of 25 μl with the following reaction components, including the final concentrations: 19.4 μl water, 2.5 μl PCR buffer (1×; containing 1.5 mM MgCl₂; ABgene, Surrey, UK); 0.5 μl dNTPs (0.2 mM); 0.5 μl forward primer (0.4 μM); 0.5 μl reverse primer (0.4 μM) and 0.1 μl polymerase Thermoprim plus (0.5 U; Abgene) according to the manufacturer’s protocol. The template of aromatase was amplified for 35 cycles including an initial denaturation step at 94°C for 3 min. Each cycle consisted of 94°C for 45 s followed by an annealing temperature for aromatase of 62°C for 45 s and elongation at 72°C for 45 s. A final elongation step at 72°C for 2 min was added. Quantity and integrity of fetal gonadal cDNA were checked by the PCR of GAPDH within 25 cycles at an annealing temperature of 60°C for the GAPDH primer set. An aliquot (7 μl) of each reaction mixture was finally resolved by agarose gel electrophoresis (1.5% (w/v)), containing 1% ethidium bromide and was visualized under u.v. light with a video documentation system (Biometra, Göttingen, Germany). Reactions containing no template (water) or non-reverse transcribed RNA (negative controls) were included at the same time to exclude contamination from buffers and tubes and to permit detection, and to exclude contamination with genomic DNA respectively.

**Real-time PCR**

Online PCRs were carried out using the real-time PCR QuantitTect SYBR Green kit protocol (Qiagen), except that the total volume was only 25 μl. Each Master mix was prepared to the indicated end concentrations: 10.7 μl water; 0.4 μl forward primer (0.32 μM); 0.4 μl reverse primer (0.32 μM); 12.5 μl 2× QuantiTect SYBR Green PCR Master Mix (1×). From the Master mix, 24 μl were filled in plastic tubes and 1 μl PCR template (20 ng reverse transcribed total RNA) was added. Quantitative fluorescence real-time RT-PCR analysis of GAPDH and aromatase was performed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were set as follows: first taq polymerase was activated at 95°C for 15 min. The amplification segment consisted of denaturation at 95°C for 15 s, followed by 20s annealing at 60°C for GAPDH and 62°C for aromatase, and extension for 30 s at 72°C. Fluorescence data were acquired after each extension step by SYBR Green binding to the amplified dsDNA at 72°C for 5 s. To evaluate specific mRNA amplification a final melting step was added by slow heating from 60 to 95°C with a rate of 0.5°C/s under continuous fluorescence measurement and a final cooling step to 40°C. Subsequent gel electrophoretic separation of PCR product was performed additionally to verify single product formation.

For the negative controls, water and non-transcribed RNA were used instead of cDNA.

The relative expression of each gene was calculated using the cycle threshold (Ct) method by setting the fluorescence threshold manually at 0.125 (ABI Sequence Detector programme software version 1.1). The Ct is the number of PCR cycles when the fluorescence signal of the specific amplicon exceeds background fluorescence.

**Statistical analysis**

Data are expressed as the arithmetic means ± s.e.m. All data were tested for normal distribution by the Kolmogorov–Smirnov Test. For testosterone and LH, the data were transformed logarithmically. The one-way analysis (ANOVA) of the statistical package for the social
The changes in aromatase mRNA expression were assayed by normalization to the GAPDH internal control. In order to obtain the Ct difference, the data for mRNA were analyzed using the Ct method described previously by Livak and Schmittgen (2001), where \( \Delta Ct = Ct_{\text{target}} - Ct_{\text{GAPDH}} \) and where \( \Delta\Delta Ct = \Delta Ct_{\text{group a, as control}} - (\Delta Ct_{\text{group b-z}}) \). The statistical analysis was based on \( \Delta\Delta Ct \) values \( (n=4 \text{ samples/group}) \). Expression changes \( (\Delta\Delta Ct) \) for the different groups were defined as follows: \( E = 10^{(-1/\text{slope})} \). From resulting cDNA standard curves, PCR efficiency \( (E) \), slope \( (s) \), and regression \( (r) \) were determined as follows: \( E=1.88, s=-3.635 \), \( r=0.99 \) for GAPDH and \( E=1.95, s=-3.373 \), \( r=0.99 \) for aromatase.

### Results

The development of testis growth in relation to overall body growth is shown in Fig. 1 from 6-week fetuses to neonates. Growth of the testis and whole body as well revealed a rather linear growth during the period investigated. However, absolute growing velocity differed. When compared with week 6, neonates had a total body weight gain by a factor of 65, whereas testis weight only increased by a factor of 6. More detailed aspects of testicular development are presented in Table 1 (morphology) where the change in tubular diameters and the data for testicular cell counts are summarized along fetal development.

Leydig cell number increased significantly after week 6. The most remarkable increase occurred around week 10. The tubular diameter increased significantly at weeks 10 and 13. As expected, the lowest diameter was found at week 6 when no Sertoli cells could be detected by the GATA-4 staining. Thereafter, the mean Sertoli cell population was around nine cells per tubule without much variation till week 13. Only at week 15, the number of Sertoli cells was significantly lower than at week 13 and in neonates. A significant threefold increase in the number of germ cells (gonocytes) was found from weeks 6 to 10. Thereafter, the number decreased at week 13. It increased again at week 15 and decreased remarkably postnatally.

At all stages of development, considerable endocrine activity of the fetuses could be quantified. Thereafter, FSH concentrations remained low between weeks 6 and 10 but increased slowly. LH concentrations were not determined at week 6 due to limited availability of plasma. They were low during fetal development but increased remarkably postnatally. High testosterone concentrations were measured at week 6 but thereafter concentrations remained at a low level of about 1.39 nmol/l. A twofold increase was found from week 15 to neonates. Total estrogens decreased from weeks 6 to 10 but thereafter they increased continuously till week 15. From the end of the pregnancy to neonates, however, the concentrations of estrogens dropped again by a factor of 3. Separate evaluation of two randomly chosen blood samples from weeks 10, 13, and 15 with an assay specific for 17β-estradiol revealed that concentrations of estradiol represent 80% of total estrogens in week 10, 15% in week 13, and 7% in week 15.

Also the expression of aromatase was significantly elevated at week 15. Quantification of aromatase expression was based on immunostaining (compare Fig. 2) and revealed that a high percentage of the two relevant cell types (gonocytes and Leydig cells) was positively stained. Table 1 gives the absolute number of cells (see morphology) and the immunostained cells (see aromatase). As additionally shown in Fig. 3, the main site of estrogen synthesis changed during fetal development. Both Leydig cells and germ cells seem to contribute to estrogen formation already at the age of 6 weeks. Thereafter, at week 10, gonocytes were transiently the main source of aromatase, whereas at the same time aromatase expression by Leydig cells was reduced. Thereafter, a continuous increase of aromatase was

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**Table 1** (morphology)

<table>
<thead>
<tr>
<th>Weeks of gestation</th>
<th>Testis weight [mg]</th>
<th>Body weight [g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>65</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>300</td>
</tr>
<tr>
<td>13</td>
<td>90</td>
<td>900</td>
</tr>
<tr>
<td>15</td>
<td>60</td>
<td>1200</td>
</tr>
<tr>
<td>Birth</td>
<td>1500</td>
<td>1500</td>
</tr>
</tbody>
</table>

**Figure 1** Development of testis (■) and body (—he) weight in fetal and neonate male pigs. Developmental stages represent means from four neonates, and five (week 6) or six (weeks 10–15) fetuses.
observed for Leydig cells, but gonocytes decreased expression continuously till the end of fetal development. An immunocytochemical example for the change of aromatase expression is also given in Fig. 2.

The aromatase expression was also confirmed by quantitative RT-PCR and revealed an increase in aromatase-mRNA along fetal development (Table 1, bottom).

**Discussion**

The demonstration of aromatase localization in different cell types of testes was demonstrated for several species during postnatal development (e.g., Conley et al., 1996, Carreau et al., 1999). In most species, aromatase activity was clearly attributed to Leydig cells. In addition, aromatase is expressed in germ cells in mouse, brown bear, and rooster (Carreau et al., 1999). A change of aromatase expression was demonstrated in early postnatal rats, where aromatase is expressed in Sertoli cells but its expression shifts to Leydig cells in mature rats (Tsai-Morris et al., 1985). The pig is unique due to its high amounts of estrogens (Claus et al., 1985), which are exclusively synthesized in Leydig cells of mature boars (Mutembei et al., 2005). The present study presents first data on aromatase localization by immunocytochemistry in fetal pigs.

It was shown earlier that considerable amounts of estrogens are measurable in plasma of umbilical artery of pigs on days 60, 75, 95, and 112 of gestation (Ford & Christenson, 1986). The concentrations were in an order as also found in the present study. We could additionally quantify the increase in testicular aromatase expression both by immunocytochemistry and by RT-PCR along fetal development. The data also show that gonocytes transiently become a major source of aromatase at week 10, whereas simultaneously expression in Leydig cells was low. At that time immature Leydig cells enter the next wave of mitogenic activity (Van Vorstenbosch et al., 1984) and thus cannot synthesize estrogens.

In postnatal piglets, FSH and LH concentrations were higher when compared with 15-week fetuses. Additionally in postnatal pigs, testosterone was elevated whereas estrogens revealed a steep decrease. Testosterone also exceeds estrogens fivefold at week 6 where aromatase transcription is low as well. It was shown earlier that

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**Table 1** Characterization of fetal testis development in the pig by morphological parameters, hormone concentrations in fetal umbilical artery plasma, and immunocytochemical and quantitative PCR characterization of aromatase expression.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>6 (n = 5)</th>
<th>10 (n = 6)</th>
<th>13 (n = 6)</th>
<th>15 (n = 6)</th>
<th>1 Day postpartum (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leydig cells (cells/mm²)</td>
<td>2948±385a</td>
<td>4121±296b</td>
<td>4676±187b</td>
<td>4663±420b</td>
<td>3670±353ab</td>
</tr>
<tr>
<td>Tubular diameter (µm)</td>
<td>51.34±1.19a</td>
<td>67.83±2.43b</td>
<td>63.22±1.61b</td>
<td>52.02±3.07a</td>
<td>57.28±2.27ab</td>
</tr>
<tr>
<td>Sertoli cells (cells/tube)</td>
<td>–</td>
<td>8.71±0.69ab</td>
<td>10.25±0.15a</td>
<td>8.48±0.56a</td>
<td>10.35±0.34a</td>
</tr>
<tr>
<td>Gonocytes (cells/tube)</td>
<td>0.76±0.10a</td>
<td>2.85±0.18abc</td>
<td>1.95±0.19b</td>
<td>2.95±0.24c</td>
<td>1.30±0.13a</td>
</tr>
<tr>
<td><strong>Hormones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH (pmol/l)</td>
<td>12.00±0.33a</td>
<td>12.33±1.33a</td>
<td>13.33±1.00a</td>
<td>19.33±1.00b</td>
<td>23.33±2.67b</td>
</tr>
<tr>
<td>LH (pmol/l)</td>
<td>–</td>
<td>7.00±2.99abc</td>
<td>3.67±1.00a</td>
<td>3.00±0.67a</td>
<td>29.33±14.33b</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>6.98±0.87a</td>
<td>1.39±0.03b</td>
<td>1.35±0.07bc, c</td>
<td>1.28±0.07b</td>
<td>2.36±0.52c</td>
</tr>
<tr>
<td>Estrogen (nmol/l)</td>
<td>1.51±0.11a</td>
<td>0.70±0.15a</td>
<td>7.50±0.88b</td>
<td>31.03±4.85bc, c</td>
<td>11.87±3.20ab</td>
</tr>
<tr>
<td><strong>Aromatase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leydig cells (cells/mm²)</td>
<td>879±35a</td>
<td>1070±57a</td>
<td>1686±116b</td>
<td>2400±44f</td>
<td>2415±138bc</td>
</tr>
<tr>
<td>Gonocytes (cells/tube)</td>
<td>0.13±0.05ad</td>
<td>1.74±0.14b</td>
<td>0.63±0.12abc, c</td>
<td>0.32±0.09d</td>
<td>0.40±0.07abc, d</td>
</tr>
<tr>
<td>mRNA (ΔCt)</td>
<td>0.00±0.66a</td>
<td>0.19±0.23a</td>
<td>1.23±0.60a</td>
<td>2.93±0.53b</td>
<td>1.70±0.41a</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. Values in rows with different superscripts are significantly different (P<0.05 to P<0.001).

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**Figure 2** Examples of immunocytochemical staining of aromatase (brown color) in week 6 (A), week 10 (B), week 13 (C), and week 15 (D) in testes of fetal pigs. Examples of germ cells expressing aromatase during week 10 are marked with arrows (B). Additionally, the negative control of aromatase (E) and the immunocytochemical staining of GATA-4 (F, top) with its negative control (F, bottom) are included. Scale bar represents 20 µm.
around day 35 of gestation masculinization of the external and internal genitalia occurs (Patten 1948, Marrable 1971). The increase of estrogens specifically at weeks 13 and 15 is also paralleled by a corresponding increase of aromatase mRNA expression, whereas the changes of estrogen concentrations and aromatase activity do not reveal an obvious relationship to FSH. Similarly, low FSH concentrations during that period had also been reported by others (Mc Coad et al. 2003).

The high concentrations of estrogens in umbilical artery plasma during the last trimester are paralleled by a similar course of estrogen concentrations in amniotic fluid (Robertson et al. 1985) and in the urine of sows along pregnancy (Velle 1958). However, these concentrations were attributed to steroid formation and aromatization in placental tissue (e.g., Rombauts 1964, Knight 1994) and do not require contribution by the fetus. It is well known that estrogens have a luteotropic function in the pig (Flint et al. 1978, Magness et al. 1983) so that it is not excluded that testicular estrogens from the fetus may contribute to maintain pregnancy. Estrogens might additionally be an anabolic agent for fetal growth, because we found that progenitor cells for myogenesis express high concentrations of estrogen receptor $\alpha$ during fetal development (unpublished results).

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Figure 3 Relative contribution of germ cells (– – –) and Leydig cells (– –) to aromatase expression. Note the transient shift of aromatase expression from Leydig cells to germ cells in week 10.


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