Evidence that High Variation in Antral Follicle Count
during Follicular Waves is Linked to Alterations in
Ovarian Androgen Production in Cattle

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ABSTRACT

Androgens have an important role in ovarian follicular growth and function, but circulating androgen concentrations are also associated with ovarian dysfunction, cardiovascular disease and metabolic disorders in women. The extent and causes of the variation in androgen production in individuals, however, are unknown. Because thecal cells of follicles synthesize androstenedione and testosterone, variation in production of these androgens is hypothesized to be directly related to the inherently high variation in number of healthy growing follicles in ovaries of individuals. To test this hypothesis, we determined whether thecal CYP17A1 mRNA (codes for a cytochrome P450 enzyme involved in androgen synthesis), LH-induced thecal androstenedione production, androstenedione concentrations in follicular fluid, and circulating testosterone concentrations were lower in cattle with relatively low versus a high number of follicles growing during follicular waves and whether ovariectomy reduced serum testosterone concentrations. Results demonstrated that cattle with a low follicle number had lower (P<0.05) abundance of CYP17A1 mRNA in thecal cells, reduced (P<0.01) capacity of thecal cells to produce androstenedione in response to LH, lower (P<0.01) androstenedione concentrations in ovulatory follicles, and lower (P<0.02) circulating testosterone concentrations during estrous cycles compared with animals with high follicle numbers. Also, serum testosterone in cattle with low or high follicle numbers was reduced by 63 and 70%, respectively, following ovariectomy. In conclusion, circulating androgen concentrations are lower in cattle with low versus a high
number of follicles growing during follicular waves possibly because of a reduced responsiveness of thecal cells to LH.

**INTRODUCTION**

Androgens provide substrate for estradiol production, which is crucial for development and function of ovulatory follicles and thus reproductive success (King* et al.* 2007, Walters* et al.* 2008). In addition, circulating androgen concentrations are associated with ovarian disorders such as polycystic ovarian syndrome (Dumesic* et al.* 2007), cardiovascular health (Wu & von Eckardstein 2003) and metabolic disease in women (Padmanabhan* et al.* 2006, Chen* et al.* 2008). While the primary tissue sources of androgens in healthy, non-pregnant females are the ovaries and adrenal glands, the extent and causes of the variation in androgen production among individuals are unknown. Because thecal cells of growing follicles synthesize two potent androgens, androstenedione and testosterone, variation in production of these androgens (by individual follicles), and correspondingly in circulating androgen concentrations (from the total pool of follicles), is hypothesized to be directly related to the inherently high variation in total number of healthy growing follicles in ovaries of individuals.

Previous studies from our laboratories show that serial ovarian ultrasonography can be used to reliably identify cattle that consistently have low or high numbers of follicles during waves (Burns* et al.* 2005, Ireland* et al.* 2007) and that the variation in the peak antral follicle count (AFC) is positively associated with size
of the ovarian reserve (total number of morphologically healthy follicles and oocytes in ovaries) (Ireland et al. 2008), but inversely associated with secretion of gonadotropins during the estrous cycle (Burns et al. 2005, Ireland et al. 2008, Ireland et al. 2009, Mossa et al. 2010). Specifically, young adult cattle with low AFC have chronically high circulating LH concentrations (Jimenez-Krassel et al. 2009) and this could lead to an enhanced production of testosterone by thecal cells. However, the capacity to produce progesterone in response to LH is lower for luteinized granulosal cells isolated from dominant follicles and for luteal cells of animals with low versus high AFC, despite similar amounts of LH receptor binding sites, indicating a possible disruption of the LH signaling system in cattle with a relatively low number of follicles growing during follicular waves (Jimenez-Krassel et al. 2009).

Consequently, to test the aforementioned hypothesis, the present study used the bovine AFC model to determine whether 1) abundance of thecal CYP17A1 mRNA, which codes for a member of the cytochrome P450 superfamily of enzymes involved in androgen synthesis, 2) androstenedione production by thecal cells in vitro, 3) concentrations of androstenedione in follicular fluid (FF) of ovulatory follicles, and 4) circulating testosterone concentrations during estrous cycles were greater in cattle with high versus a low AFC, and 5) whether serum testosterone concentrations decreased following ovariectomy and removal of the ovarian reserve in cattle.

RESULTS
Alterations in CYP17A1 mRNA in thecal cells of cattle with low versus a high AFC

As previously reported (Ireland et al. 2009), diameters of the three largest follicles per pair of ovaries combined collected during the first 2 to 3 d of the follicular wave were similar (P>0.05; 6.1±0.3 vs 5.4±0.3 mm) for animals in the low versus high AFC groups. However, analysis of mRNA in thecal cells from these follicles showed that the abundance of CYP17A1 mRNA was 60% lower (P<0.05) in cells isolated from cattle with low versus a high AFC (0.74± 0.22 versus 1.83±0.61). FF samples were exhausted after completion of other assays thus androgen concentrations in FF could not be determined in this study.

Alterations in basal and LH-induced androstenedione production by thecal cells from antral follicles of cattle with low versus a high follicle number

Although there was no difference in basal androstenedione production, LH-stimulated androstenedione production was 2.5 fold higher (P<0.05) for thecal cells isolated from cattle in the high compared with the low follicle number group (Fig 1).

Alterations in intrafollicular concentrations of androstenedione in ovulatory follicles of cattle with low versus a high AFC
Within 12 h after the PG injection to induce luteolysis and dominant follicle development, diameter (15.74±0.36 vs 14.74±0.42 mm) and progesterone concentrations in FF (123±14 vs 101±7 ng/ml) of ovulatory follicles were similar (P>0.05) for animals in the low versus high AFC groups. In contrast, intrafollicular concentrations of androstenedione were 77% (P<0.03, 32.67± 6.45 vs 109.55±25.87 ng/ml) and estradiol were 26% (P<0.01; 435± 59 vs 588±76 ng/ml) lower in FF aspirated from ovulatory follicles of animals with low versus a high AFC.

Alterations in serum testosterone concentrations during estrous cycles of beef heifers and dairy cows with low versus a high AFC

Serum testosterone concentrations varied (P<0.01) during the days of the estrous cycles of both beef heifers and non-lactating dairy cows. Although testosterone concentrations were lower in dairy cows (0.04±0 vs 0.10±0.01), patterns of testosterone secretion were similar for animals with low or a high AFC (data not shown). Nevertheless, animals with a low AFC had an overall lower (P<0.02) serum concentration of testosterone between Days 0 to 14 of the estrous cycle for beef heifers and between Days 6 to 21 for non-lactating dairy cows compared with animals with a high AFC (Fig. 2). In addition, serum testosterone concentrations were lower (P<0.05) on Days 2 (0.03±0.01 vs 0.09± 0.03ng/ml)) and 3 (0.03±0.01vs 0.08±0.03 ng/ml) of the estrous cycle in beef heifers and on Days 8 and 12 to 16 of the cycle in non-lactating dairy cows (Fig. 2) for animals in the low versus high AFC group.
Alterations in serum testosterone concentrations after ovariectomy of cattle with low versus a high AFC

The average testosterone concentrations decreased (P<0.0001) by 70% and 63% by 9 days after ovariectomy in the high and low AFC groups, respectively, compared to overall average for testosterone concentrations before ovariectomy collected on Day 1, 5 and 10 of the estrous cycle (Day 0). Testosterone concentrations were not different before or after surgery between the high and low AFC groups (Fig 3).

DISCUSSION

The most significant finding of the present study is that ovarian androgen production is positively associated with the number of antral follicles ≥3 mm in diameter. Several diverse lines of evidence strongly support this conclusion. Firstly, in cattle with low AFC the abundance of thecal CYP17A1 mRNA, which codes for a cytochrome P450 enzyme involved in androgen synthesis, responsiveness of thecal cells to LH treatment and FF concentrations of androstenedione in ovulatory follicles were diminished compared to cattle with a high AFC. Secondly, serum testosterone concentrations were lower overall during estrous cycles of young adult beef cattle and older dairy cows with low versus a high AFC. Thirdly, surgical removal of ovaries, and thereby the ovarian reserve, markedly reduced serum testosterone concentrations in cattle. Taken
together, these findings imply that follicular androgen production by thecal cells is linked positively to number of follicles growing during follicular waves and correspondingly to size of the ovarian reserve in cattle, which explains why ovarian androgen production is relatively low during estrous cycles for cattle with low versus a high AFC.

Several key questions arise from these novel findings. Firstly, why is androgen production inversely associated with LH secretion? Secondly, what is the impact, if any, of the differences in ovarian androgen production between cattle with low or high AFC on fertility and on other physiological systems?

We previously demonstrated that cattle with low versus high AFC have a 2-fold greater basal LH concentration (Jimenez-Krassel et al. 2009), whereas the present work shows that both circulating testosterone concentrations during the estrous cycle and LH-induced androstenedione production by theca cells are lower in cattle with a low compared to high AFC. Considering that steroidogenic activity of thecal cells is mainly regulated by LH (Li et al.) and that the inherent capacity of the pituitary gland to produce gonadotropins is not influenced by AFC (Mossa et al. 2010), this discrepancy could be due to a reduced responsiveness of thecal cells to LH for cattle with a low AFC. This hypothesis is confirmed by the recent finding that the capacity of both luteinized granulosal cells isolated from dominant follicles and of luteal cells isolated from CL on Day 12 of the estrous cycle to produce progesterone basally and in response to LH are lower for cattle with low versus a high AFC (Jimenez-Krassel et al. 2009). Moreover, two recent studies showed that LH significantly increased androstenedione production in
bovine thecal cells (in accordance with our results) and that addition of two inhibitors of the PI3K/Akt signaling pathway significantly decreased LH-induced androstenedione production (Fukuda et al. 2009), inhibition of the Akt and Erk pathway inhibited both basal and LH-induced androstenedione secretion by bovine theca cells \textit{in vitro} (Ryan et al. 2008). Recent evidence shows that LH significantly increases CYP17A1 mRNA expression in thecal cells (Fukuda et al. 2009) and in the present study CYP17A1 mRNA expression was higher in thecal cells from cattle with a high AFC, despite lower basal and episodic LH secretion (Jimenez-Krassel et al. 2009). Therefore, the reduced androgen production in animals with a low AFC could be due to an alteration in LH signaling, which in turn, may alter CYP17A1 mRNA expression.

We have recently shown that intrafollicular estradiol concentration in the three largest follicles (5-7 mm) at emergence of the follicular wave is greater in cattle with low versus a high AFC (Ireland et al. 2009). In contrast, intrafollicular estradiol concentrations were lower in ovulatory follicles (14-15 mm) in cattle with low versus a high AFC in the present study. While several explanations are possible, this difference in estradiol production by the two different follicle types could be due to the reduced responsiveness of dominant compared with smaller antral follicles to LH in cattle with a low AFC. In support of this possibility, it is well established that granulosal and thecal cells of dominant follicles develop LH receptors in sheep and cattle (Webb & Campbell 2007). Therefore, the lower sensitivity of granulosal and thecal cells to LH in cattle with low versus a high AFC could also explain the lower intrafollicular estradiol and androstenedione
concentrations at the ovulatory, LH-dependent stage of follicular development, as observed in the present study.

Ovarian AFC is inversely associated with circulating FSH concentrations during the estrous cycle (Burns et al. 2005, Ireland et al. 2008, Ireland et al. 2009, Mossa et al. 2010) and there is evidence suggesting a reciprocal control between androgens and ovarian sensitivity to FSH. For example, in vivo treatment with testosterone increases androgen receptor mRNA in granulosal and thecal cells in small antral follicles of primates, and the expression of androgen receptor mRNA is positively correlated with FSH receptor (FSHR) mRNA expression (Weil et al. 1999). In addition, dihydrotestosterone (DHT) increases FSHR expression in preovulatory follicles in gilts (Cardenas et al. 2002) and administration of testosterone or DHT to female rhesus monkeys and pregnant ewes (prenatally treated fetuses) stimulates primordial follicle initiation and increases growing follicle numbers and overall follicle survival (Vendola et al. 1999, Steckler et al. 2005, Forsdike et al. 2007). During oocyte maturation and ovulation, androgens increase abundance of FSHR mRNA and number of preovulatory follicles and corpora lutea (Walters et al. 2008). Testosterone increases murine follicle responsiveness to FSH (Wang et al. 2001) and androstenedione stimulates FSH-mediated differentiation of bovine granulosal cells, as indicated by an increase in aromatase activity and estradiol production (Hamel et al. 2005). In women, serum androgen levels are correlated with the ovarian response to FSH, as assessed by the peak estradiol levels and the number of follicles and number of oocytes and embryos retrieved (Barbieri et al.
227 2005), and significant positive correlations exist between the AFC and both
228 baseline and stimulated steroid serum concentrations (Hugues et al. 2010).
229 Therefore, cattle with a high AFC and higher serum androgen concentrations
230 could have an enhanced ovarian sensitivity to FSH and this could in turn explain
231 why a greater number of follicles are recruited per wave, despite the lower serum
232 concentrations of FSH compared to cattle with a lower AFC. Moreover, we have
233 recently demonstrated that the inherent capacity of the pituitary gland to produce
234 gonadotropins is not different between cattle with high versus low numbers of
235 follicles during follicular waves (Mossa et al. 2010), implicating ovarian factors as
236 the more plausible causes of FSH variation.
237
238 The possible effects of elevated gonadotropins and relatively low
239 androgen concentrations on fertility are unknown. In women ovarian ageing is
240 characterized by a reduced ovarian sensitivity to FSH, which is accompanied by
241 a progressive decline in serum androgen levels (Davison et al. 2005, Sowers et
242 al. 2009). Several studies show that women with an elevated FSH concentration,
243 independent of age, have a poor response to ovarian stimulation, leading to a
244 lower pregnancy rate using assisted reproduction techniques (Scott et al. 1989,
245 Martin et al. 1996, Sharif et al. 1998, El-Toukhy et al. 2002). However, Abadalla
246 and Thum (Abdalla & Thum 2004) report that elevated basal FSH concentrations
247 are not indicative of deterioration of oocyte and embryo quality and explained the
248 reduction in pregnancy rate as a result of a reduced number of oocytes collected
249 and the subsequently limited choice of embryos available for transfer in women
250 undergoing IVF treatment. Cattle with a low AFC show similarities with ageing
women, such as increased secretion of FSH (Burns et al. 2005, Ireland et al. 2008) and reduced serum progesterone (Jimenez-Krassel et al. 2009) and AMH during the estrous cycle (Ireland et al. 2008), reduced ovarian reserve (Ireland et al. 2008), poor response to superovulation (Ireland et al. 2007), and, as our present results show, reduced androgen concentrations during estrous cycles. These findings may suggest an impaired fertility in cattle with low AFC. However, to date no studies have investigated the association between AFC and reproductive success in cattle. Specifically, the possible effects of the variation in androgen concentrations on reproduction are unknown. For example, the addition of exogenous testosterone (100 nM) to oocyte maturation medium, selected on the basis of physiological concentrations reported in preovulatory bovine follicles (~165 nM (Dieleman et al. 1983)), improved the developmental competence of bovine oocytes after in vitro fertilization (Younis et al. 1989, Silva & Knight 2000). On the other hand, hyperandrogenism is among the main features of polycystic ovarian syndrome (PCOS), one of the most common causes of ovulatory infertility in women (Pasquali & Gambineri 2006). Present results also indicate ovaries as the primary source of androgens in female cattle, regardless of number of follicles. Serum testosterone concentrations did not differ significantly between the two groups of cattle with low or high follicle numbers before the removal of the ovaries probably because blood was sampled on Day 1, 5 and 9 of the estrous cycle when the differences in testosterone concentrations are not statistically significant, as shown in the
present study. Nevertheless, results of ovariectomy and removal of the ovarian reserve confirm that the ovary is the main source of androgens in cattle.

In conclusion, this study has demonstrated for the first time that responsiveness of thecal cells to LH is lower in cattle with low versus a high number of antral follicles growing during follicular waves as assessed by the reduced capacity of thecal cells to produce androstenedione in response to LH, lower abundance of CYP17A1 mRNA in thecal cells, lower androstenedione concentrations in ovulatory follicles, and diminished circulating testosterone concentrations during estrous cycles. Further studies will be necessary to investigate the possible effects of the variation in the ovarian reserve, and associated androgen production, on fertility as well as the causes of the high variation in numbers of follicles and oocytes in ovaries of mammals.

MATERIALS AND METHODS

Animals

Cattle used in experiments were located at the Michigan State University Beef or Dairy Cattle Teaching and Research Centers or Lyons Research Farm, University College Dublin, Ireland. All experiments were performed in compliance with protocols approved by the All University Committee on Animal Use and Care at Michigan State University, or the Animal Research Ethics Subcommittee, University College Dublin, the Cruelty to Animal Act (Ireland, 1876), and the European Union Directive 86/609/EC.
Identification of cattle with consistently low versus a high AFC during follicular waves

Ovaries of each animal were monitored with an Aloka SSD-900 ultrasound machine using a linear array trans-rectal probe (7.5-MHz transducer) and follicles were counted as previously described (Burns et al. 2005, Ireland et al. 2007, Ireland et al. 2008, Ireland et al. 2009). Each antral follicle ≥ 3 mm in diameter was drawn on an ovarian map and diameter and total number of follicles ≥ 3 mm in diameter per pair of ovaries, hereafter referred to as AFC, was recorded for each animal. Cattle that consistently had a relatively low versus high peak AFC during follicular waves were identified as previously explained (Burns et al. 2005).

Briefly, a group of adult animals of similar ages and weights were injected two or three times with prostaglandin F$_2\alpha$ (PG) spaced 11 d apart to initiate luteolysis and synchronize occurrence of ovulation. Ovaries were then subjected to once or twice daily ovarian ultrasonography to determine AFC beginning on Days 1 to 2 after the last PG injection and continuing until completion of the study. Ovulation day was defined as the first day that the preovulatory follicle was not observed by ultrasonography. Peak AFC was determined for 3 to 5 follicular waves per animal, and the average peak value for AFC per wave was used to classify cattle into the low (≤ 15 follicles) or high (≥ 25 follicles) AFC groups (Burns et al. 2005, Ireland et al. 2007, Ireland et al. 2008, Ireland et al. 2009, Mossa et al. 2010). Animals in the intermediate category (16-24 follicles) were not studied further and were returned to the herd.
In previously published studies (Ireland et al. 2007, Ireland et al. 2009, Mossa et al. 2010), 177 cross-bred beef heifers and 116 dairy cows were examined and 13% (n = 39) were ranked in the low, 76% (n = 223) in the intermediate and 11% (n = 31) in high AFC group. The number of animals used in each experiment is specified below.

**Alterations in CYP17A1 mRNA in thecal cells of cattle with low versus a high AFC**

Thirty-two cross-bred beef heifers (Hereford × Angus × Charolais, 11–13 mo old) were initially used for this experiment and heifers were assigned to one of two groups as described above: low (≤15 follicles, n = 5 animals) or high (≥25 follicles, n = 5 animals). Remaining animals in the intermediate category (n = 22) were excluded. Thecal cells were obtained from each of the three largest growing follicles per pair of ovaries during the early stages of the first follicular wave on Days 2 to 3 of the estrous cycle for adult beef heifers (n = 3 largest follicles per pair of ovaries for 5 animals per group) with low versus a high AFC as explained previously (Ireland et al. 2009). Abundance of CYP17A1 mRNA was measured in thecal cells as explained below.

**Alterations in basal and LH-induced androstenedione production by thecal cells from dominant follicles of cattle with low versus a high number of follicles**
Thecal cells were obtained from first wave dominant follicles (Ireland et al. 1980) isolated from ovaries obtained at an abattoir from cattle with high (≥25 follicles ≥ 3 mm in diameter, n = 3 animals) or low (< 15 follicles ≥ 3 mm in diameter, n = 3 animals) follicle numbers. The theca interna was dissected from the follicle, scraped clean of granulosal cells and cut into 8 pieces. Pieces of theca (2 per well) from each animal were then separately cultured for 24 h with 0 or 4 ng/ml of bLH (NIH AFP-11743B), as previously described (Lv et al. 2009). After culture, media was aspirated and stored at -20°C until determination of androstenedione concentration, and the theca pieces were snap frozen in liquid nitrogen and stored at -80°C. The theca pieces were then lysed by homogenization and sonication in ice-cold RIPA buffer (Sigma, St. Louis, MO) plus Complete Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN). Lysates were centrifuged (10,000 x g for 10 min at 4°C) and protein in supernatant was determined with use of the DC Protein Assay (Bio-Rad, Hercules, CA). Data were expressed as ng androstenedione per mg protein.

Alterations in intrafollicular concentrations of androstenedione in ovulatory follicles of cattle with low versus a high AFC

Fifty-five cross-bred beef heifers (12-14 mo of age) were examined and animals with low (n=7), intermediate (n=38) and high (n=10) AFC were identified as previously described; heifers in the intermediate group were excluded from the experiment and returned to the herd. On day 7 of the estrous cycle during the first follicular wave, PG was injected and 12 h later the diameter of the ovulatory
follicle was measured using ultrasonography and FF was collected from the
dominant ovulatory follicle (largest) via ultrasound-guided follicular aspiration
(Jimenez-Krassel et al. 2003). Granulosal cells were separated by centrifugation
and FF was frozen within 15 min of collection. After thawing, concentration of
androstenedione, progesterone and estradiol were determined for each aspirated
FF supernatant.

Alterations in serum testosterone concentrations during estrous
cycles of beef heifers and dairy cows with low versus a high AFC

As previously reported (Ireland et al. 2007), 90 cross-bred beef heifers (11
to 12 mo of age) were segregated into the low (n = 14), intermediate (n = 65),
and high (n= 11) AFC groups. A subset of heifers with low (n = 7 animals) versus
a high (n = 7) AFC were synchronized with two injections of PG given 11 d apart.
Daily blood samples were collected from the second PG to Day 14 of the estrous
cycle. In a second previously published study (Mossa et al. 2010), 116 non-
lactating Holstein cows (4 to 8 yrs of age) were examined and cattle with low (n =
5 animals) versus a high (n = 4) AFC were selected and synchronized with two
injections of PG given 11 d apart. After the second PG injection, daily blood
samples were collected from Day 6 to 21 of the estrous cycle. Blood samples
from these two previously published studies (Ireland et al. 2009, Mossa et al.
2010) were used as the source of serum samples for testosterone analysis in the
present study.
To investigate the contribution of the ovaries in animals with high or low AFC to circulating androgen concentrations, this study determined whether ovariectomy altered serum testosterone concentrations in cattle with low or high AFC. The same non-lactating Holstein cows (4 to 8 yrs of age) with low (n = 5 animals) or a high (n = 4) AFC used in a previous paper (Mossa et al. 2010) and for serum testosterone determinations as explained above were also used in this study. Testosterone concentrations were determined in blood samples taken on Days 5 and 10 of the estrous cycle. In addition, estrous cycles were synchronized with two injections of PG spaced 11 days apart. Ten days later cows received 2 additional injections of PG 16 h apart to ensure rapid luteolysis; and, 22-28 h after the 4th PG injection, cows were bilaterally ovariectomized via colpotomy (Drost et al. 1992). Blood samples were collected 1 h before and 1 and 9 days after ovariectomy to monitor alterations in serum testosterone concentrations.

**Hormone Analyses**

Total testosterone in serum was measured following ether extraction of serum samples (0.5 ml) using commercially available RIA kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA; Cruinn Diagnostics Limited, Dublin, Ireland). Inter- and intra-assay CVs were < 13% and limit of detection was 0.03 ng/ml.
Concentrations of androstenedione, progesterone and estradiol in FF or media were analyzed using commercially available kits from Siemens Medical Solutions Diagnostics. Intra-assay CVs were < 10% for each assay.

**mRNA analysis**

Total RNA was extracted from thecal cells of each follicle and processed for analysis by real-time PCR as previously explained (Ireland et al. 2009). Primers for bovine CYP17A1 were forward 5’- CAAGGATGGCAACCTGAAGT - 3´ and reverse 5’- GAGAGAGAGGCTCGGACAGA - 3´. Abundance of CYP17A1 mRNA was normalized against levels of the constitutively expressed housekeeping gene ACTB and relative amounts of gene expression were calculated using the formula $2^{-(\Delta\Delta Ct)}$ (Livak & Schmittgen 2001).

**Statistical Analyses**

Testosterone concentrations in serum of intact animals, and before and after ovariectomies were analyzed using Proc Mixed procedure of SAS (SAS 1995). Data were examined for normality of distribution with the Shapiro-Wilk test, and when heterogeneity of variance was detected, data were natural log-transformed before analyses, but actual values are reported. The model included the effects of group (low vs high), day of estrous cycle and their interaction. Day of blood sampling was treated as a repeated measure across individual animals. When main effects were significant, a Bonferroni-t test was used to determine if statistical significant ($P \leq 0.05$) differences existed among individual means.
Concentrations of steroids in FF or media and abundance of thecal mRNA for CYP17A1 were statistically analyzed using the Proc Mixed model of SAS (SAS 1995). Data were examined for normality of distribution as described above. Main effects included animal groups (low versus high). When main effects were significant (P < 0.05), a Bonferroni-t test was used to determine if statistical significant (P < 0.05) differences existed among individual means.

Declaration of interest: there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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FIGURE LEGENDS

**Fig. 1.** Alterations in basal and LH-induced thecal production of androstenedione in cattle with low versus high AFC.

Androstenedione production by theca interna isolated from first wave dominant follicles of ovaries from animals with low (n = 3) or a high (n = 3) AFC. Theca interna (2 pieces per well) from each follicle were cultured in duplicate for 24 h with or without 4 ng/ml LH as previously described (Lv et al. 2009). Androstenedione production was normalized to total protein contained in thecal tissue after culture. Each bar represents the mean ± SEM for each follicle number group. Asterisk indicates significant (P<0.05) difference between means.

**Fig. 2.** Alterations in serum testosterone concentrations during estrous cycles of dairy cows with a low versus high AFC.

Concentration of testosterone in serum in Holstein cows with low (open symbols, n = 5 animals) versus a high (solid symbols, n = 4) AFC. Animals were synchronized with two injections of PG given 11 d apart, and blood samples were collected daily from Day 6 to 21 of the estrous cycle. Ovulation occurred on Day
1 of the estrous cycle, and ovulation was defined as the day of disappearance of
largest dominant follicle as determined by ovarian ultrasonography. ANOVA
indicated there were highly statistically significant (P<0.02) differences in
testosterone concentrations during the estrous cycle (Day) and between AFC
groups (Group). Asterisks indicate significant (P<0.05) difference between
means. Each symbol represents the daily mean ± SEM for animals in each AFC
group.

Fig. 3. Alterations in serum testosterone concentrations after ovariectomy
of cattle with low versus a high AFC.

Circulating concentrations of testosterone were measured in Holstein
cows with low (open symbols, n= 5 animals) versus high (solid symbols, n = 4)
AFC. Animals were synchronized with PG and blood samples taken at various
times before (Day 5 and 10 of estrous cycle and 1 h before ovariectomy) and 1
and 9 d after ovariectomy as explained in Methods. The zero time point
represents the pooled mean ± SEM for Day 5 and 10 of the estrous cycle and
one h before ovariectomy for cows in the low or high AFC groups. Asterisks
indicate significant (P< 0.01) difference from Day 0 values.