Testosterone stimulates progesterone production and *STAR*, *P450* cholesterol side-chain cleavage and LH receptor mRNAs expression in hen (*Gallus domesticus*) granulosa cells

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Abstract

The chicken ovary is organized into a hierarchy of yellow yolky follicles that ovulate on successive days. Active or passive immunization of laying hens against testosterone blocks ovulation without affecting follicle development. Testosterone may play a role in pre-ovulatory follicle maturation by stimulating granulosa progesterone production. We assessed whether this stimulus is dose-related and depends on the maturity of the donor follicle, and if it does so by stimulating granulosa cell *STAR*, *P450* cholesterol side-chain cleavage (*P450scc*), and LH receptor (*LHCGR*) mRNAs expression. Progesterone production by granulosa cells from F1, F3, and F4 follicles, cultured for 3 h without testosterone was greater in cells collected 11–14 h than 1–4 h after ovulation. These differences in progesterone production were less pronounced after granulosa cells had been cultured for 24 h. Culture of granulosa cells for 3 or 24 h with testosterone (1–100 ng/ml) stimulated progesterone production in cells collected from F4, F3, or F1 follicles 1–4, or 11–14 h after ovulation. Testosterone (0–4000 ng/ml) alone or in combination with LH (0–100 ng/ml) increased progesterone production by F1 granulosa cells, collected 1–4 and 11–14 h after ovulation and cultured for 3 h. Finally, testosterone (10 or 100 ng/ml) increased *STAR*, *P450scc*, and *LHCGR* mRNAs, when added to 3 h cultures of F1 granulosa cells. In conclusion, testosterone stimulates granulosa cell progesterone production in hen pre-ovulatory hierarchical follicles irrespective of maturational state, acting alone or additively with LH. We propose that testosterone promotes granulosa cell maturation to facilitate the pre-ovulatory release of LH.

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Introduction

In the domestic hen, the pre-ovulatory release of LH is stimulated by the positive feedback action of progesterone (Johnson *et al.* 1985) and is associated with a pre-ovulatory release of testosterone, the functional significance of which is not fully understood (Wilson & Cunningham 1984, Etches 1994). The ovarian source of the pre-ovulatory surge of progesterone is primarily the granulosa cell layer of the mature pre-ovulatory follicle (F1), while the pre-ovulatory release of testosterone originates from all hierarchical pre-ovulatory follicles (Etches & Duke 1984). Injection of testosterone can induce ovulation associated with a pre-ovulatory release of progesterone, but only if the injection results in unphysiologically high concentrations of circulating testosterone (Croze & Etches 1980). Because passive immunization with testosterone antibodies blocks spontaneous ovulation (Furr & Smith 1975) and the pre-ovulatory release of testosterone is initiated before that of progesterone (Etches & Cunningham 1977, Williams &

Sharp 1978, Wilson & Cunningham 1984), it has been suggested that the pre-ovulatory release of testosterone may act to prime the hypothalamo-pituitary axis to facilitate the pre-ovulatory release of LH (Croze & Etches 1980). We have shown that active or passive immunization of laying hens against testosterone blocks ovulation without affecting the development of hierarchical pre-ovulatory follicles (Rangel *et al.* 2005), and that treatment of laying hens with flutamide, a testosterone antagonist, blocks the pre-ovulatory surges of both progesterone and LH and ovulation (Rangel *et al.* 2006). Furthermore, testosterone (0.1–10 ng/ml), acting alone, or with LH stimulates progesterone production by cultured granulosa cells from mature pre-ovulatory (F1) follicles (Rangel *et al.* 2007). This observation is consistent with the presence of androgen receptors in these cells (Yoshimura *et al.* 1993). Our finding confirmed that of Sasanami & Mori (1999) who demonstrated that 1 µg/ml of testosterone stimulated progesterone output by cultured quail granulosa cells and increases their sensitivity to LH. Taken together, these observations suggest that the

pre-ovulatory release of testosterone may facilitate the pre-ovulatory release of progesterone from granulosa cells in the F1 pre-ovulatory follicle.

Other investigators have found an inhibitory effect of testosterone on progesterone production (Johnson *et al.* 1988, Lee & Bahr 1989, 1990), so it is therefore possible that the stimulatory effect of testosterone on granulosa cell production is dose related, being stimulatory with physiological doses and inhibitory with supra-physiological doses. Furthermore, it is possible that the stimulatory effects of testosterone on granulosa cell progesterone production may be related to the maturation of the donor pre-ovulatory follicle. Thus, in this study we tested three hypotheses, first, that the stimulatory action of testosterone on hen granulosa cell progesterone production depends on the degree of maturation of the donor follicle (i.e. collection time post-ovulation; 1–4 or 11–14 h). Second, that testosterone stimulates granulosa cell progesterone production in a dose related manner. Finally, that the stimulatory action of testosterone on F1 hen granulosa cell progesterone production is mediated by increased *STAR*, *P450* cholesterol side-chain cleavage (*P450_{scc}*), and LH receptor (*LHCGR*) expression, as reflected in increases in their encoding mRNAs.

Results

Effect of testosterone, follicle size, and collection time post-ovulation on progesterone production by pre-ovulatory granulosa cells after culture for 24 h

Testosterone stimulated progesterone production by F1 granulosa cells at all doses tested ($P < 0.01$), with the exception of granulosa cells from F1 follicles collected 11–14 h after ovulation and cultured with 1 ng/ml testosterone. No differences were observed between progesterone production by granulosa cells from F1 follicles that were obtained between 1–4 and 11–14 h after ovulation ($P > 0.05$; Fig. 1A). Testosterone stimulated progesterone production by F3 follicle granulosa cells at all doses ($P < 0.01$), with no differences between 1 vs 10 and 10 vs 100 ng/ml ($P > 0.05$; Fig. 1B). When F3 follicle granulosa cells were cultured without testosterone, progesterone production by cells collected 1–4 h after ovulation was lower than by cells collected 11–14 h after ovulation ($P < 0.01$), but no differences between the two collection times were observed when testosterone was added to the cell cultures (Fig. 1B). Testosterone stimulated progesterone production by F4 granulosa cells in a dose related manner ($P < 0.01$) up to 10 ng/ml ($P > 0.05$; Fig. 1C). Progesterone production by F4 granulosa cells was lower in cells collected 1–4 h, than in cells collected 11–14 h after ovulation when they were cultured without testosterone ($P < 0.01$; Fig. 1C), and this difference disappeared in the presence of 10 or 100 ng/ml testosterone (Fig. 1C). In the absence of testosterone, progesterone production by F4 granulosa cells was significantly lower than that by F1 and F3 granulosa cells ($P < 0.01$), but no differences in

Figure 1 Progesterone production by hen granulosa cells cultured for 24 h with testosterone (0, 1, 10, or 100 ng/ml). Granulosa cells were obtained from (A) F1, (B) F3, or (C) F4 follicles, at 1–4 h (diamonds, dashed line) or 11–14 h (squares, solid line) after ovulation. Values are least square means $\pm$ S.E.D. (error bar) for the difference between testosterone concentrations within the same follicle size and time after ovulation. For comparison at the same level of testosterone treatment and different time of collection, the S.E.D. = 0.65. Mean values with different letters differ significantly within a collection time (a, b, c for 1–4 h and x, y, z for 11–14 h after ovulation). Nine cultures were prepared from F1, five from F3 and four from F4 follicles at both times after ovulation, each culture contained 24 replicates per treatment.

progesterone production were observed between F1 and F3 granulosa cells cultured without testosterone ($P > 0.05$). Nonetheless, when testosterone was added to the culture, the differences in progesterone production between follicle size disappeared ($P > 0.05$; Fig. 1A and B), with the exception of F4 cells, where 1 ng/ml testosterone stimulated less progesterone production than from F3 granulosa cells ($P < 0.01$; Fig. 1B and C).

Effect of testosterone, follicle size, and collection time post-ovulation on progesterone production by granulosa cells after culture for 3 h

Progesterone production by F1 granulosa cells collected 1–4 h after ovulation were stimulated by a high concentration (100 ng/ml) of testosterone ($P < 0.01$). In contrast, progesterone production by cells collected 11–14 h after ovulation showed a stimulatory response to a tenfold lower testosterone concentration (10 ng/ml; $P < 0.01$; Fig. 2A). Progesterone production by F3 granulosa cells was lower in cells collected 1–4 h after ovulation, than in cells collected 11–14 h after ovulation, irrespective of whether the cells were cultured with or without testosterone ($P < 0.01$; Fig. 2B). In F3 granulosa cells collected 1–4 h and 11–14 h after ovulation progesterone production was stimulated by 10 and 100 ng/ml testosterone ($P < 0.01$; Fig. 2B), but in cells coming from follicles 11–14 h after ovulation no differences were observed between 10 and 100 ng/ml testosterone ($P > 0.05$). Progesterone production by F4 granulosa cells was significantly lower in cells collected 1–4 h after ovulation ($P < 0.01$) than in cells collected 11–14 h after ovulation independently of the addition of testosterone (Fig. 2C). In F4 granulosa cells, testosterone increased progesterone production in a dose related manner ($P < 0.01$; Fig. 2C). However, in cells collected 11–14 h after ovulation no differences in progesterone production were observed between doses of 10 and 100 ng/ml of testosterone ($P > 0.05$). When cells were culture for 3 h without testosterone, there were differences in granulosa cell progesterone production between cells collected at different maturational stages ($P < 0.01$; Fig. 2). However, this difference was not seen in F1 and F3 when cells were culture for 24 h (Fig. 1).

Effect of low or high concentrations of testosterone, collection time post-ovulation, and LH on progesterone production by F1 granulosa cells after culture for 3 h

Progesterone production by hen F1 granulosa cells cultured with testosterone and LH was higher in cells collected 11–14 h after ovulation than in cells collected 1–4 h after ovulation ($P < 0.05$; Table 1). Testosterone alone or with 1 ng/ml LH increased progesterone production by F1 granulosa cells, irrespective of time of collection ($P < 0.05$; Table 1). LH stimulated a significant increase in progesterone production when added at 10 and 100 ng/ml ($P < 0.05$), irrespective of the time when cells were collected (Table 1). When testosterone was added to the culture medium together with 10 and 100 ng/ml of LH, testosterone did not further stimulate progesterone production in cells collected 11–14 h after ovulation ($P > 0.05$). Testosterone at the highest dose used (4000 ng/ml) in the presence of 10 but not 100 ng/ml LH (Table 1), depressed

Figure 2 Progesterone production by hen granulosa cells cultured for 3 h with testosterone (0, 1, 10 or 100 ng/ml). Granulosa cells were obtained from (A) F1, (B) F3, or (C) F4 follicles, 1–4 h (diamonds, dashed line) or 11–14 h (squares, solid line) after ovulation. Values are means $\pm$ s.e.d. (error bar) for the difference between testosterone concentrations within the same follicle size and time after ovulation. For comparison at the same level of testosterone treatment and different time of collection the s.e.d. = 1. Mean values with different letters differ significantly within a collection time (a, b, c for 1–4 h and x, y, z for 11–14 h after ovulation). Four cultures were prepared for each follicle size (F1, F3, and F4), at both times after ovulation with 24 replicates per treatment.

progesterone production by granulosa cells collected at 11–14 h, but not 1–4 h after ovulation.

Effect of testosterone and collection time post-ovulation on mRNA expression for STAR, P450scc, and LHCGR by pre-ovulatory granulosa cells after culture for 3 h

Testosterone significantly increased *STAR*, *P450scc*, and *LHCGR* mRNAs in F1 granulosa cells after culture for 3 h at the two doses used (10 and 100 ng/ml;

Table 1 Progesterone production (natural logarithm of pg of progesterone production by 10 000 cells) by hen F1 granulosa cells cultured for 3 h with testosterone and LH. Granulosa cells were obtained 1–4 or 11–14 h after ovulation.

LH concentration in culture media (ng/ml)	Testosterone concentration (ng/ml) in culture media					
	0	10	100	1000	2000	4000
Follicles collected 1–4 h after ovulation						
0	4.017	4.452	4.323	5.474*	6.042*	6.703*
1	4.228	4.345	4.283	5.204*	6.105*	6.644*
10	6.691	6.559	6.589	7.114*	6.934	6.879
100	6.815	6.967	6.979	7.214*	7.168	6.985
Follicles collected 11–14 h after ovulation						
0	5.591	5.627	5.747	5.966	6.553*	7.011*
1	5.403	5.482	5.598	5.755	6.497*	6.665*
10	8.484	8.374	8.362	8.529	8.378	8.072*
100	8.624	8.522	8.651	8.423	8.361	8.249

For comparison of different testosterone concentrations within the same LH concentration the s.e.d. = 0.09. *Significant differences for comparisons against 0 ng/ml of testosterone within the same LH concentration. Four cultures were prepared for each time after ovulation, with four replicates per treatment.

$P < 0.001$; Fig. 3A–C). There were no differences due to testosterone dosage nor time after ovulation at which the granulosa cells were collected. The data collected from granulosa at 1–4 and 11–14 h after ovulation were therefore pooled (Fig. 3). The testosterone induced increase in *STAR*, *P450scc* and *LHCGR* expression was associated with increased progesterone production ($P < 0.01$) with no differences between doses of testosterone (Fig. 3D).

Discussion

This study confirms our previous finding (Rangel *et al.* 2007) that testosterone stimulates progesterone production by granulosa cells from the F1 follicle, and extends it by showing that testosterone also stimulates progesterone production by granulosa cells harvested from the less mature, F3 and F4, follicles in the follicular hierarchy. This effect of testosterone is therefore not related to the maturity of the donor follicle. We also show that this stimulatory effect of testosterone on granulosa cell progesterone production is associated with increased *STAR*, *P450scc* and *LHCGR* mRNAs. The augmented expression of these genes involved in the steroidogenesis explains the increase in progesterone production seen after testosterone treatment.

Our studies support a stimulatory paracrine action of testosterone produced by theca cells on *STAR*, *P450scc*, and *LHCGR* expression in granulosa cells. The presence of *STAR* mRNA in F1 granulosa cells agrees with earlier studies (Johnson & Bridgham 2001) and with the view that it is necessary to generate the pre-ovulatory surge of progesterone (Johnson *et al.* 2002). Recently, Nakao *et al.* (2007) showed, in Japanese quail, that *STAR* expression in the F1 follicle is clock-driven, and is stimulated by LH. These observations suggest that *STAR* play a key role in the circadian control of steroidogenesis in the F1 follicle and consequently in the circadian

timing of ovulation. We propose that testosterone may act in both independently and in an additive manner with LH to stimulate *STAR* expression in granulosa cells.

P450scc is an essential enzyme for steroidogenesis and increased expression of *P450scc* in granulosa cells plays a key role in the recruitment of follicles into the pre-ovulatory follicular hierarchy (Tilly *et al.* 1991a, 1991b). The testosterone-induced increase in *P450scc* expression observed in our study supports our view that the increase in progesterone production by hen granulosa cells of the pre-ovulatory F1 follicle is partly testosterone-dependent. However, this conclusion is not consistent with an earlier study showing that testosterone inhibits granulosa cell *P450scc* activity (Lee & Bahr 1990). These authors observed an inhibitory effect of testosterone on *P450scc* activity using doses (10 μ M) that were much greater than those used in our studies (0.34 μ M maximum). This suggests that our observations may be more physiologically meaningful, assuming that increased *P450scc* expression results in increased *P450scc* enzyme activity.

The observation that testosterone increased *LHCGR* mRNA in F1 follicle granulosa cells may explain why LH receptor mRNA increases in granulosa cells during follicle maturation (Johnson *et al.* 1996, Zhang *et al.* 1997, Yamamura *et al.* 2001). Increased granulosa *LHCGR* accounts for the progressive increase in responsiveness of granulosa cells to LH in maturing follicles (Bahr & Johnson 1984). Testosterone stimulation of *STAR*, *P450scc*, and *LHCGR* expression in the granulosa cells of the F1 follicle is therefore consistent with a major role for testosterone in controlling follicular maturation.

The finding that testosterone stimulates progesterone production by granulosa cells from mature and immature pre-ovulatory hierarchical follicles requires that we reinterpret our earlier findings that active, or passive immunization against testosterone blocks ovulation

Figure 3 Effect of testosterone (0, 10, or 100 ng/ml) added to 3 h cultures of granulosa cells taken from F1 follicles 1–4 h and 11–14 h (combined) after ovulation on amounts of mRNA for (A) *STAR*, (B) *P450scc*, (C) *LHCGR*, and (D) on progesterone production. Data collected for the 1–4 and 11–14 h collection times were pooled since there were no differences between them. Values are means $\pm$ S.E.D. (0.038) of logarithm progesterone production by 10 000 cells, or logarithm of number of cDNA copies by microgram of RNA $\pm$ S.E.D. (0.086 for *STAR*, 0.080 for *P450scc* and 0.136 for *LHCGR*). Mean values with different letters differ significantly ($P < 0.01$). Three cultures were prepared with eight replicates per treatment.

(Rangel *et al.* 2005) and that a specific testosterone antagonist, flutamide, acutely blocks the pre-ovulatory surge of progesterone in laying hens (Rangel *et al.* 2006). We suggested that the response of granulosa cells to testosterone was related to the developmental stage of the hierarchical follicles being inhibitory in immature follicles, whereas in mature follicles testosterone may 'prime' the granulosa cells making them more responsive to LH to initiate the pre-ovulatory release of progesterone. This action of testosterone was first suggested by Croze & Etches (1980). Active or passive immunization against testosterone, or flutamide treatment was suggested to block ovulation by preventing this 'priming' effect. However, we later showed that testosterone directly enhances the production of progesterone by F1 granulosa cells and acts additively with LH (Rangel *et al.* 2007).

The stimulatory action of testosterone on granulosa cell progesterone synthesis may contribute to the

progressive increase in progesterone in granulosa cells seen in follicles as they develop from the F4 to F1 stage (Bahr *et al.* 1983). However, some suppressive factor is required to prevent this increase in granulosa cell progesterone being too rapid and thereby inducing premature follicular maturation. This factor is likely to be thecal cell estrogen since this steroid also inhibits granulosa cell progesterone production *in vitro* (Johnson *et al.* 1988, Lee & Bahr 1989, 1990). Furthermore, estrogen production is highest in the smaller follicles in the yellow yolk follicular hierarchy and progressively decreases to very low values in the mature F1 pre-ovulatory follicle (Bahr *et al.* 1983, Etches & Duke 1984). It is therefore possible that estrogen originating from thecal cells counteracts the role of testosterone, to suppress progesterone production by granulosa cells in immature pre-ovulatory hierarchical follicles in a paracrine manner.

Basal and testosterone-stimulated progesterone production by granulosa cells collected from F1, F3, and F4 follicles 1–4 or 11–14 h after ovulation and cultured for 24 h was similar (Fig. 1). However, when the cells were cultured for only 3 h, it became clear that basal and testosterone-stimulated progesterone production was markedly higher in cells collected 11–14 h after ovulation (Fig. 2). This observation is consistent with earlier studies showing that granulosa cell progesterone production in all pre-ovulatory hierarchical follicles begins to increase progressively from about 6 h after ovulation (Shahabi *et al.* 1975, Bahr *et al.* 1983, Etches & Duke 1984).

As reported by Shahabi *et al.* (1975) progesterone production by F3 granulosa cells before or after a pre-ovulatory surge of LH is higher than in cells collected from either F4 or F1 follicles and that progesterone production by the F4 and F1 cells is similar. Similarly, a study performed in hen granulosa cells, to evaluate the induction of progesterone production in response to insulin-like growth factor 1, reported a fourfold increase in progesterone secretion by pooled F3 and F4 granulosa cells and only a 1.5-fold increase in F1 granulosa cells (Tosca *et al.* 2008). Nonetheless, others have suggested that granulosa cells from F3 to F5 follicles are less responsive to LH stimulation than F1 and F2 follicles (Robinson *et al.* 1988). Furthermore, Yu *et al.* (1992) found that the F1 pre-ovulatory follicle produced 30 times more progesterone *in vitro* than the F2 to F5 follicles in the absence of exogenous bLH. Our observations support that the F3 follicle is the most steroidogenically active for progesterone production in the presence of basal concentrations of LH.

The increased responsiveness of immature granulosa cells in hierarchical pre-ovulatory follicles to the stimulatory action of testosterone on progesterone production 11–14 h after ovulation may account for the initiation of the progressive increase in granulosa progesterone production in all follicles after each ovulation

(Bahr *et al.* 1983, Etches & Duke 1984). In addition, as an avian follicle changes its maturational stage in such a short time-frame, cell-culture studies in avian granulosa cells should be short-term; otherwise the physiological differences between follicles could be masked by maturation of the cells *in vitro*, as shown here in the 24 h culture.

In conclusion, testosterone stimulates granulosa cell progesterone production in hen pre-ovulatory hierarchical follicles irrespective of maturational state. This action of testosterone is a consequence of a stimulatory effect of testosterone on granulosa cell *STAR*, *P450_{scc}*, and *LHCGR* mRNA expression. It is suggested that a stimulatory paracrine effect of thecal testosterone may play a role in increasing progesterone production by granulosa cells in the maturing pre-ovulatory follicle and that this is countered by an inhibitory paracrine action of thecal estrogen in the less mature follicles. The development of the F1 follicle is characterized by decreasing estrogen production which is suggested to decrease its inhibitory action on granulosa cell progesterone production. Granulosa cell progesterone production in the F1 follicle may be enhanced by the stimulatory action of increasing circulating testosterone originating from less mature follicles in the hierarchy, to initiate the pre-ovulatory release of progesterone. This in turn stimulates the positive feedback action of progesterone on LH release to generate the respective pre-ovulatory surges.

Materials and Methods

Reagents

Dulbecco's PBS (DPBS 10×) and M199 (medium 199 with Earle's salts) were purchased from Gibco BRL Life Technologies. Hepes, trypan blue, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), *N,N*-dimethylformamide, hydrochloric acid, trypsin type II, pen-strep (containing 10 000 IU penicillin and 10 mg streptomycin/ml), 2,2 α -azino-bis(3-ethylbenz-thiazoline acid), sodium hydroxide 1 M, citric acid, EDTA, sodium azide, sodium phosphate dibasic, sodium phosphate monobasic, hydrofluoric acid, acetic acid, Tween 20 (polyoxyethylene-sorbitan monolaurate), BSA (fraction V), insulin (bovine pancreatic insulin), testosterone, and sodium chloride were purchased from Sigma Chemical Co. Ltd. SDS was purchased from Bio-Rad. Newborn calf serum was purchased from Equitech-Bio Inc. (Ingram, TX, USA), and was heat-treated at 60 °C for 10 min to destroy steroid-binding proteins that could interfere with steroid treatments in culture or steroid measurements. Progesterone, testosterone, and estradiol concentrations were undetectable in denatured serum. Ninety-six-well culture plates (Nunclon Surface and MaxiSorp Surface, Nunc-Immuno plates) were purchased from Nunc Brand Products (Roskilde, Denmark). Bottle-top filters (0.22 μ m cellulose acetate) and cell-culture dishes (60×15 mm polystyrene and 35×10 mm polystyrene) were purchased from Corning Inc. (Corning, NY, USA). RNAqueous-Micro, Micro Scale RNA Isolation Kit from Ambion Inc.

(Austin, TX, USA). SuperScript First-Strand Kit was purchased from Invitrogen Corp. Sterile water was purchased from Pisa SA de CV (Mexico). Agarose low melting point was purchased from Promega. QIAquick gel extraction Kit was purchased from Qiagen. TaqMan Universal PCR probes and Master Mix, no AmpErase UNG were made from Applied Biosystems (Foster City, CA, USA).

Ovine LH (oLH 26 NIADDK) was obtained from the National Hormone and Peptide Program.

Experimental animals

Animal procedures were approved by the Ethics and Animal Welfare Committee of the Veterinary Faculty, UNAM. Leghorn laying hens in the middle of their first laying cycle (42 weeks old) were housed in individual cages with water and food *ad libitum*, under a 14 h light:14 h darkness (14L:14D) schedule to synchronize the time of ovulation within the flock (Leeson *et al.* 1979). An ovarian follicle acquires the capacity to ovulate about 10 h after ovulation of the formerly F1 largest follicle in the ovary and ovulation occurs within a few minutes of oviposition (Etches 1994). Animals were killed by cervical dislocation to collect pre-ovulatory follicles when the F1 follicles were predicted to be immature or mature, 1–4 and 11–14 h after ovulation/oviposition respectively. F3 and F4 follicles were collected at the same time.

Granulosa cell culture

Pre-ovulatory hierarchical follicles (F1, F3, and F4) from each animal were placed individually in cell culture dishes containing sterile DPBS at room temperature. The granulosa cell layer was separated from the theca as previously described (Gilbert *et al.* 1977, Rangel *et al.* 2007). Briefly, the yolk was drained out through an incision made in the follicular wall, after which the follicle was inverted and shaken in sterile physiological solution. The granulosa layer detached from the follicle was recovered from the bottom of the solution. Once isolated, granulosa cells were disaggregated at 37 °C for 15 min under continuous agitation in 5 ml of digesting solution containing 1 mg/ml of trypsin type II in DPBS. After incubation, cells were dispersed by flushing them with a Pasteur pipette. The enzymatic solution was quenched by the addition of 1 ml of new born calf serum. Cells were washed by centrifugation during 3 min at 500 *g*, at 22 °C. After isolation granulosa cell pellets were resuspended in culture medium (medium M199 containing 1.1 g of Hepes, 10% of new born calf serum, 100 IU/ml of penicillin, 75 IU/ml of streptomycin, and 10 ng/ml of insulin), and viable cell number was determined with a haemocytometer using trypan blue dye exclusion at 0.4%. Granulosa cells were plated out in 96-well culture plates at a final concentration of 50 000 viable cells per well (experiments 1, 2 and 3) and 100 000 viable cells per well in 250 μ l of culture medium (experiment 4). Cells were cultured in a humidified atmosphere with 5% CO₂ and 95% air at 41 °C.

In the first experiment, granulosa cells were cultured for 24 h and quantified by MTT staining at the end of culture (Berridge & Tan 1993). Briefly, after removal of 175 μ l culture media supernatant at the end of culture, 20 μ l MTT solution

Table 2 Primers and probe sequences and size of the amplicons generated by real-time RT-PCR assay for hen STAR, P450_{osc}, and LH receptor.

Target	Forward primer (5'–3')	Reverse primer (5'–3')	TaqMan probe (5'–3')	Amplicon (pb)	Genbank identification
STAR-chicken	TCAGCCGCGCGGATTAAAGG	TGGTGGCTGCTACAAACACT	FAM-CTGCTGCCTCAGCCCCC	63	NM_204686
P450 _{osc} -chicken	ACCGTGACTACCGCAACAAG	AGGCCTCCCTGTCTTGA	FAM-CCTACGGCGCTGCTCC	53	NM_001001756
LHCGR chicken	GCGCTGTGCCTGCAC	CACAGGAGGTGAGTGAATGAC	FAM-CCAGCCCCGCCCTGC	96	AB009283

Primers and probes were designed using Primer Express 3.0 software (Applied Biosystems, CA, USA) and chicken sequences in GenBank, and synthesised by Applied Biosystems.

(5 mg MTT per 1 ml of culture medium) were added and incubated for 24 h. Viable cells metabolised the MTT to formazan, turning them blue (Slater *et al.* 1963). Formazan was released from the cells by lysing them with 100 µl lysis buffer (5 ml distilled water, 5 ml *N*-*N*-dimethyl-formamide, 250 µl HCl 1 M, 250 µl acetic acid 0.25 M, and 1 g SDS, pH 4.7) at room temperature for 4 h. Cell number was directly related to the absorbance of the medium when read on a spectrophotometer at 630 nm. A regression equation was fitted to calculate the relationship between absorbance and cell number. Cell quantification after MTT staining reflects the number of cells present per well at the end of the culture period, as we have observed that cell proliferation of hen granulosa cells does not occur *in vitro*. In addition, it has been reported that granulosa cell proliferation in pre-hierarchical follicles is low, and in the fast growing phase of the hierarchical follicles, increased diameter is achieved by reorganization of the originally multilayer to a monolayer granulosa compartment (Johnson 1993, 1996). Furthermore, Tischkau & Bahr (1996) reported a negative relationship between follicle progesterone production and cell proliferation in granulosa cells.

All other experimental cultures were for 3 h and cell number was taken as being equal to number of seeded cells.

Progesterone measurements

Progesterone production in culture was measured in the culture media supernatant by ELISA (Munro & Stabenfeldt 1984, Rangel *et al.* 2007). The sensitivity of the assays was 4.7 pg/well. The intra-assay coefficient of variation (CV) was between 2 and 11% and the inter-assay CV was between 6 and 12% for the low and the high quality controls respectively.

The concentration of progesterone was expressed as progesterone produced by 10 000 cells.

Progesterone antibodies, standards and conjugates for the ELISA were kindly provided by Dr C Munro (Davis University, CA, USA). Progesterone antibody cross reactivity was 21 and 29% against 11 α -hydroxyprogesterone and 5 α -pregnene-3, 20-dione, and below 0.5% for other steroids (Munro & Stabenfeldt 1984).

Experimental design

Four experiments were conducted using cultured granulosa cells. The first experiment determined the effect of collection time post-ovulation (1–4 and 11–14 h), follicle size (F1, F3, and F4 follicles) and testosterone (0, 1, 10, and 100 ng/ml) on progesterone production by granulosa cells in 24 h in culture. Nine hens were killed and their F1, F3, and F4 follicles dissected out. Granulosa cell cultures for F1 follicles were performed with cells coming from a single follicle. Granulosa cells from F3 and F4 were pooled for every two follicles to yield sufficient number of cells when needed, resulting in five cultures performed for F3 and four for F4 follicles at both times after ovulation. Each culture contained 24 replicates (culture wells) per treatment. For experiments 2 to 4, F1, F2, and F3 follicles were obtained from a every hen and each culture was performed with granulosa cells coming from a single ovarian follicle. The second experiment determined the effect of

collection time post-ovulation (1–4 and 11–14 h), follicle size (F1, F3, and F4 follicles), and testosterone (0, 1, 10, and 100 ng/ml) on progesterone production by granulosa cells after 3 h in culture. Eight hens were used to prepare four cultures for each follicle size (F1, F3 and F4), at both times after ovulation with 24 replicates per treatment. The third experiment determined the effect of collection time post-ovulation (1–4 and 11–14 h), oLH (0, 1, 10, and 100 ng/ml) and low (0, 10, and 100 ng/ml) or high (1000, 2000, and 4000 ng/ml) concentrations of testosterone on progesterone production by F1 granulosa cells after 3 h in culture. Eight hens were used to prepare four cultures were prepared for both times after ovulation with four replicates per treatment. The final experiment determined the effect of testosterone (0, 10, and 100 ng/ml) on *STAR*, *P450scc*, and *LHCGR* mRNAs and progesterone production by F1 granulosa cells, collected 1–4 and 11–14 h after ovulation and cultured for 3 h. Six hens were used to prepare three cultures were performed with eight replicates per treatment. At the end of culture, cells were pooled within treatment, and total RNA extracted by RNeasy-Micro, RNA isolation Kit (Ambion); thus $n=3$. RT was performed on 1 µg total RNA preparation using Oligo (dT) primer and RT, as recommended by the manufacturer (SuperScript First-Strand Kit, Invitrogen). *STAR*, *P450scc*, and *LHCGR* mRNAs in granulosa cells were quantified by real-time PCR using the primers shown in Table 2. Amplification cycles were 50 °C for 2 min, 95 °C for 10 min and then ran for 45 cycles at 95 °C for 15 s, 60 °C for 1 min using TaqMan Universal PCR Master Mix.

The amounts of *STAR*, *P450scc*, and *LHCGR* mRNAs expressed in granulosa cells were estimated from standard cDNA curves prepared for each gene. Briefly, a pool of the total cDNA was made taking 4 µl of each sample, and amplified in a PCR reaction using the same primers as for the real time PCR, at 94 °C for 5 min and then run for 40 cycles at 94 °C for 15 s and 60 °C for 1 min. The amplification products were separated by electrophoresis in a 2.5% agarose low melting point gel and identified using a transilluminator. The amplification products were extracted from the gels using the QIAquick gel extraction Kit (Qiagen), and quantified densitometrically using a spectrophotometer. Total cDNA copies were calculated as described by Tricarico *et al.* (2002). The relationship between quantitative PCR (C_t) values and cDNA copies in 1 µg RNA were determined by regression analyses, which were found to be linear. The number of cDNA copies for the unknown samples was calculated from the regression equation. Each standard curve was made in a range of 1×10^{10} – 1×10^3 copies.

Statistical analyses

The first experiment was an unbalanced design and analysed by restricted Maximum Likelihood with collection time post-ovulation, follicle size, and testosterone as fixed effects and culture as a random effect. Data for this study are presented as least square means $\pm$ S.E.D. Experimental designs for experiments 2 and 3 were arranged as split-split-plot design, where the collection time post-ovulation was the main plot factor, follicle size (experiment 2) or LH concentration (experiment 3) was the subplot factor, and testosterone concentration was the

sub-subplot factor. For experiment 4 the design was a split-plot design where the collection time post-ovulation was the main plot factor and testosterone concentration within the collection time post-ovulation was the subplot factor. Data for these studies are presented as means $\pm$ S.E.D.; Kuehl 2001).

To correct for heterogeneity of variance, data were analyzed after logarithmic transformation of progesterone produced by 10 000 cells, or logarithmic transformation of cDNA copies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Bahr JM & Johnson AL 1984 Regulation of the follicular hierarchy and ovulation. *Journal of Experimental Zoology* **232** 495–500.
- Bahr JM, Wang S-C, Huang MY & Calvo FO 1983 Steroid concentrations in isolated theca and granulosa layers of preovulatory follicles during the ovulatory cycle of the domestic hen. *Biology of Reproduction* **29** 326–334.
- Berridge MV & Tan AS 1993 Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular location, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Archives of Biochemistry and Biophysics* **303** 474–482.
- Croze F & Etches RJ 1980 The physiological significance of androgen-induced ovulation in the hen. *Journal of Endocrinology* **84** 163–171.
- Etches RJ 1994 Maturation of ovarian follicles. In *Reproductive Biology of Poultry*, pp 29–49. Eds FJ Cunningham, PE Lake & D Hewitt. Cambridge: British Poultry Science Ltd.
- Etches RJ & Cunningham FJ 1977 The plasma concentration of testosterone and LH during the ovulation cycle of the hen (*Gallus domesticus*). *Journal of Endocrinology* **71** 51–58.
- Etches RJ & Duke CE 1984 Progesterone, androstenedione and oestradiol content of theca and granulosa tissues of the four largest ovarian follicles during the ovulatory cycle of the hen (*Gallus domesticus*). *Journal of Endocrinology* **103** 71–76.
- Furr BJA & Smith GK 1975 Effects of antiserum against gonadal steroids on ovulation in the hen *Gallus domesticus*. *Journal of Endocrinology* **66** 303–304.

- Gilbert AB, Evans AJ, Perry MM & Davidson MH 1977 A method for separating the granulosa, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (*Gallus domesticus*). *Journal of Reproduction and Fertility* **50** 178–191.
- Johnson AL 1993 Regulation of follicle differentiation by gonadotropins and growth factors. *Poultry Science* **72** 867–873.
- Johnson AL 1996 The avian ovarian hierarchy: a balance between follicle differentiation and atresia. *Poultry and Avian Biology Reviews* **7** 99–110.
- Johnson AL & Bridgham JT 2001 Regulation of steroidogenic acute regulatory protein and luteinizing hormone receptor messenger ribonucleic acid in hen granulosa cells. *Endocrinology* **142** 3116–3124.
- Johnson PA, Johnson AL & van Tienhoven A 1985 Evidence for a positive feedback interaction between progesterone and luteinizing hormone in the induction of ovulation in the hen, *Gallus domesticus*. *General and Comparative Endocrinology* **58** 478–485.
- Johnson PA, Green C, Lee HT & Bahr JM 1988 Inhibition of progesterone secretion from granulosa cells by estradiol and androgens in the domestic hen. *Endocrinology* **123** 473–477.
- Johnson AL, Bridgham JT & Wagner B 1996 Characterization of a chicken luteinizing hormone receptor (cLH-R) complementary deoxyribonucleic acid, and expression of cLH-R messenger ribonucleic acid in the ovary. *Biology of Reproduction* **55** 304–309.
- Johnson AL, Solovieva EV & Bridgham JT 2002 Relationship between steroidogenic acute regulatory protein expression and progesterone production in hen granulosa cells during follicle development. *Biology of Reproduction* **67** 1313–1320.
- Kuehl RO 2001 Diseño de parcelas divididas. In *Diseño de Experimentos. Principios Estadísticos de Diseño y Análisis de Investigación*, pp 469–491. Ed. RO Kuehl. México: Thomson Learning.
- Lee HT & Bahr JM 1989 Inhibitory sites of androgens and estradiol in progesterone biosynthesis in granulosa cells of the domestic hen. *Endocrinology* **125** 760–765.
- Lee HT & Bahr JM 1990 Inhibition of the activities of P450 cholesterol side-chain cleavage and 3 β -hydroxysteroid dehydrogenase and the amount of P450 cholesterol side-chain cleavage by testosterone and estradiol-17 β in progesterone biosynthesis in hen granulosa cells. *Endocrinology* **126** 779–786.
- Leeson S, Summers JB & Etches RJ 1979 Effect of a 28 h light:dark cycle on egg shell quality in the end of lay birds. *Poultry Science* **58** 285–287.
- Munro C & Stabenfeldt G 1984 Development of a microtitre plate enzyme immunoassay for the determination of progesterone. *Journal of Endocrinology* **101** 41–49.
- Nakao N, Yasuo S, Nishimura A, Yamamura T, Watanabe T, Anraku T, Okano T, Fukada Y, Sharp PJ, Ebihara S *et al.* 2007 Circadian clock gene regulation of steroidogenic acute regulatory protein gene expression in preovulatory ovarian follicles. *Endocrinology* **148** 3031–3038.
- Rangel PL, Lassala IA & Gutierrez CG 2005 Testosterone immunization blocks the ovulatory process in laying hens without affecting ovarian follicular development. *Animal Reproduction Science* **86** 143–151.
- Rangel PL, Sharp PJ & Gutierrez CG 2006 Testosterone antagonist flutamide blocks ovulation and preovulatory surges of progesterone, luteinizing hormone and oestradiol in laying hens. *Reproduction* **131** 1109–1114.
- Rangel PL, Rodriguez A & Gutierrez CG 2007 Testosterone induces progesterone production and interacts with physiological concentrations of LH to increase granulosa cell progesterone production in hens (*Gallus domesticus*). *Animal Reproduction Science* **102** 56–65.
- Robinson FE, Etches RJ, Anderson-Langmuir CE, Burke WH, Cunningham FJ, Ishii S, Sharp PJ & Talbot RT 1988 Steroidogenic relationship of gonadotrophin hormones in the ovary of the hen (*Gallus domesticus*). *General and Comparative Endocrinology* **69** 455–466.
- Sasanami T & Mori M 1999 Effects of oestradiol-17 β and testosterone on progesterone production in the cultured granulosa cells of Japanese quail. *British Poultry Science* **40** 536–540.
- Shahabi NA, Norton HW & Nalbandov AV 1975 Steroid levels in follicles and the plasma of hens during the ovulatory cycle. *Endocrinology* **96** 962–968.
- Slater TF, Swyer B & Strauli U 1963 Studies on succinate-tetrazolium reductase system III. Points of coupling of four different tetrazolium salts. *Biochimica et Biophysica Acta* **19** 477–482.
- Tilly JL, Kowalski KI & Johnson AL 1991a Stage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. *Biology of Reproduction* **44** 305–314.
- Tilly JL, Kowalski KI & Johnson AL 1991b Cytochrome P450 side-chain cleavage (P450_{scc}) in the hen ovary II. P450_{scc} messenger RNA, immunoreactive protein and enzyme activity in developing granulosa cells. *Biology of Reproduction* **45** 967–974.
- Tischkau SA & Bahr JM 1996 Avian germinal disc region secretes factors that stimulate proliferation and inhibit progesterone production by granulosa cells. *Biology of Reproduction* **54** 865–870.
- Tosca L, Chabrolle C, Crochet S, Tesseraud S & Dupont J 2008 IGF-1 receptor signaling pathways and effects of AMPK activation on IGF-1-induced progesterone secretion in hen granulosa cells. *Domestic Animal Endocrinology* **34** 204–216.
- Tricarico A, Pinzani P, Bianchi S, Paglierani M, Distanti V, Pazzaglia M, Bustin SA & Orlando C 2002 Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single house-keeping genes is inappropriate for human tissue biopsies. *Analytical Biochemistry* **309** 293–300.
- Williams JB & Sharp PJ 1978 Control of the preovulatory surge of luteinizing hormone in the hen (*Gallus domesticus*): the role of progesterone and androgens. *Journal of Endocrinology* **69** 93–102.
- Wilson SC & Cunningham FJ 1984 Endocrine control of the ovulation cycle. In *Reproductive Biology of Poultry*, pp 29–49. Eds FJ Cunningham, PE Lake & D Hewitt. Cambridge: British Poultry Science Ltd.
- Yamamura N, Takeishi M, Goto H, Tagami M, Mizutani T, Miyamoto K, Doi O & Kamiyosi M 2001 Expression of messenger RNA for gonadotropin receptor in the granulosa layer during the ovulatory cycle of hens. *Comparative Biochemistry and Physiology. Part A, Physiology* **129** 327–337.
- Yoshimura Y, Chang C, Okamoto T & Tamura T 1993 Immunolocalization of androgen receptor in the small, preovulatory, and post ovulatory follicles of laying hens. *General and Comparative Endocrinology* **91** 81–89.
- Yu MW, Robinson FE & Etches RJ 1992 Quantification of ovarian steroidogenesis in the domestic-fowl by incubation of intact large follicles. *Poultry Science* **71** 346–351.
- Zhang C, Shimada K, Saito N & Kausaku N 1997 Expression of messenger ribonucleic acids of luteinizing hormone and follicle-stimulating hormone receptors in granulosa and theca layers of chicken preovulatory follicles. *General and Comparative Endocrinology* **105** 402–409.

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