Reproduction, Fertility and Development

Insulin promotes cat preantral follicle growth and antrum formation through temporal expression of genes regulating steroidogenesis and water transport

Journal:	*Reproduction, Fertility and Development*
Manuscript ID	RD17454
Manuscript Type:	Research paper
Date Submitted by the Author:	28-Oct-2017
Complete List of Authors:	Thongkittidilok, Chommanart; University of Colorado, Medicine, Infectious Disease Division Singh, Ram; Central Avian Research Institute, Physiology and Reproduction Division Comizzoli, Pierre; Smithsonians National Zoological Park, Department of Reproductive Sciences Wildt, David; Smithsonians National Zoo, Conservation and Research Center Songsasen, Nucharin; Smithsonians National Zoo, Conservation and Research Center
Keyword:	follice, gene expression, insulin, cell culture

Insulin promotes cat preantral follicle growth and antrum formation through temporal expression of genes regulating steroidogenesis and water transport

Short title: Insulin enhances cat follicle growth in vitro

Authors: Chommanart Thongkittidilok[1]*, Ram pratap Singh[1], Pierre Comizzoli[1], David Wildt[1], Nucharin Songsasen[1]

Address:

[1]Center for Species Survival, Smithsonian Conservation Biology Institute, National Zoological Park, 1500 Remount Road, Front Royal, Virginia 22630 and 3001 Connecticut Avenue, NW, Washington, DC, 20008, USA

***Correspondence and reprint requests:** Chommanart Thongkittidilok, Division of Infectious Diseases, University of Colorado, Anschutz Medical Campus, 12700 East 19th Avenue, Mail stop B-168, Aurora, Colorado, 80045, USA

E-mail: Chommanart.thongkittidilok@ucdenver.edu

Grant support: This research was supported by, the National Institutes of Health, Smithsonian Competitive Grant Program for Science and a fellowship gift from Dr. Clint and Missy Kelly.

Key words: cat, follicle, insulin, gene expression, in vitro culture

Abstract

The objectives were to determine the influence of insulin on in vitro (1) viability and growth of domestic cat ovarian follicles, (2) mRNA expression of genes regulating steroidogenesis (*Cyp17a1*, *Cyp19a1*, *Star*) and water transport (aquaporins (AQPs); *Aqp1*, *Aqp3*, *Aqp7*, *Aqp9*) and (3) steroid production (17β-oestradiol, E2; progesterone, P4; androstenedione, A4). Cat secondary follicles were isolated from ovarian cortices and cultured in 0 (Control), 1, or 10 μg/ml insulin for 14 d (Day 0 = culture onset). Follicle/oocyte viability (based on neutral red staining), diameter, and antrum formation were assessed every 72 h and at the end of incubation (Day 14). mRNA expression of steroidogenic and water transport genes were

evaluated on Days 0, 6, and 12, and E2, P4, and A4 concentrations in medium on Day 12. By Day 14, insulin at 1 and 10 μg/ml promoted ($P < 0.05$) antrum formation in 26.9 ± 9.0% (mean ± SEM) and 78 ± 10.2% of follicles, respectively, as well as follicle growth (diameter, 151.4 ± 4.5 and 169.9 ± 10.5 μm) compared to the Control (3.3 ± 3.3% and 129.1 ± 6.6 μm). High insulin (10 μg/ml) treatment increased ($P < 0.05$) follicle viability compared to the Control (86.0 ± 9.8 versus 38.1 ± 10.9%). However, insulin exerted no benefit ($P > 0.05$) on oocyte diameter. *Cyp17a1* expression on Days 6 and 12 was higher ($P < 0.05$) in follicles cultured in the low (1 μg/ml) compared to high (10 μg/ml) insulin treatment with both being similar to the Control. *Star* expression was higher ($P < 0.01$) in low insulin compared to the Control on Day 6, but this gene was undetectable in the high insulin treatment by Day 12. Compared to the high dosage, low insulin increased ($P < 0.05$) *Aqp1* expression on Day 6; however, differences among treatments were not observed on Day 12. By contrast, high insulin supplementation decreased ($P < 0.05$) transcript level of *Aqp9* compared to the Control. Only P4 production was influenced by insulin supplementation with concentration being higher ($P < 0.05$) in the low compared to high dose and Control groups. In summary, findings indicated that insulin promoted cat ovarian follicle growth and survival in vitro, including by enhancing antrum formation, with the likely mechanism involving temporal expression of *Cyp17a1*, *Star*, and *Aqp9* genes.

1. Introduction

The ability to artificially grow a preantral follicle to the antral stage containing a developmentally competent oocyte has enormous potential for rescuing the valuable genomes of endangered wildlife species (Comizzoli *et al.* 2009; Songsasen *et al.* 2012) and preserving fertility of women facing cancer treatments (Hovatta 2004; Barrett and Woodruff 2010). In vitro culture of ovarian tissue also offers a valuable platform for advancing our understanding of the mechanisms regulating folliculogenesis (Picton *et al.* 2008; West-Farrell *et al.* 2009). Yet, successful production of live offspring from oocytes recovered from in vitro grown follicles remains limited to only the mouse (Eppig and O'Brien 1996; Xu *et al.*

2006). Attempts to apply mouse protocols to larger-size, more complex animal models (buffalo (Gupta *et al.* 2008); pig (Wu *et al.* 2001); sheep (Luz *et al.* 2012); goat (Magalhaes *et al.* 2011); baboon (Xu *et al.* 2011)) have met with limited success in advancing follicle size and oocyte viability in vitro. Nonetheless, studying non-rodent models has provided insights into the important roles of endocrine and paracrine controls in ovarian folliculogenesis (Knight and Glister 2003; Hunter *et al.* 2004).

In our laboratory, the primary model for sorting out the complexities of folliculogenesis has been the domestic cat, because findings have application to conserving felid colonies used to study human disorders, including hypertrophic cardiomyopathy (Camacho *et al.* 2016) and ophthalmic diseases (e.g., glaucoma and neuroretinal degenerative diseases) (Narfström *et al.* 2013). Furthermore, what we have learned about the domestic cat has had relevance and indirect and direct conservation benefits to a host of endangered felid species, including the clouded leopard (Pelican *et al.* 2006), cheetah (Comizzoli *et al.* 2009; Moulavi *et al.* 2017) and African lion (Troyer *et al.* 2011). Our specific investigations into follicle culture in the domestic cat have demonstrated that protein free medium and an agarose gel system support follicular survival in vitro (Fujihara *et al.* 2012). Addition of growth factors, such as epidermal growth factor (Fujihara *et al.* 2014) or stem cell factor (Thuwanut *et al.* 2016), to ovarian cortice culture now is known to improve follicle activation and viability as well as ovarian cell proliferation.

Insulin is a peptide hormone that appears to play multiple roles in regulating folliculogenesis, oocyte maturation, and embryo development in the few mammals studied to-date *(Chaves et al.* 2011). Insulin is known to act via an insulin receptor that is widely distributed within the ovarian somatic system, including the granulosa and theca cells of the human (Willis *et al.* 1996), pig (Quesnel 1999), and cow (Shimizu *et al.* 2008). In large mammals, insulin acts synergistically with FSH to promote follicular cell proliferation and differentiation (Willis *et al.* 1996; Xu *et al.* 2009; Chaves *et al.* 2012), facilitate FSH-dependent steroidogenesis *(May et al.* 1980; Duleba *et al.* 1997), and induce LH receptor expression in granulosa cells (Silva

and Price 2002). The only work on the effect of insulin in the cat follicle dates to more than 2 decades ago when Jewgenow and Göritz (1995) determined that insulin enhances granulosa cell proliferation and proportions of oocytes with an intact germinal vesicle in cultured preantral follicles. Otherwise, the mechanisms by which insulin may be involved in preantral follicle development in the cat are unknown. Furthermore, no investigation has ever been undertaken to determine the role of this peptide hormone on water transport genes that are known to exist in ovarian follicles. We have been particularly interested in understanding factors that influence formation of the follicle's antrum. Water transport genes, including *Aqp1, Aqp3*, *Aqp7*, *Aqp8*, and *Aqp9*, have been implicated as involved in antral cavity establishment in mammals, including the mouse (Huang *et al.* 2006), pig (Skowronska *et al.* 2009), and human (Thoroddsen *et al.* 2011; Lee *et al.* 2016). Meanwhile, studies of other cell types have revealed that insulin down-regulates *Aqp1, Aqp3, Aqp7*, and *Aqp9*, including in a human colon cell line (Asai *et al.* 2006; Higuchi *et al.* 2007), fetal membranes (Bouvier *et al.* 2015), and mouse pancreatic β-cells (Louchami *et al.* 2012).

The objectives of our studies here were to examine the influence of insulin on in vitro (1) viability and growth of cat follicles, (2) mRNA expression of genes regulating steroidogenesis (Cytochrome P450 family 17 subfamily, a polypeptide 1, *Cyp17a1*; Cytochrome P450 family 19 subfamily, a polypeptide 1, *Cyp19a1*; and steroidogenic acute regulatory protein, *Star*) and water transport (aquaporins, *Aqp1*, *Aqp3*, *Aqp7*, *Aqp9*), and (3) steroidogenesis (17β-oestradiol, E2; progesterone, P4; and androstenedione, A4). We hypothesized that insulin promotes antral formation, growth, and survival in incubated cat secondary follicles, and that this effect occurs by influencing temporal expression of genes known to regulate steroidogenesis and water transport.

2. Materials and methods

All chemicals used were purchased from Sigma Aldrich (St. Louis, Missouri, USA) unless stated otherwise.

2.1 Collection of ovaries

Fresh ovaries were obtained from domestic cats as recently described by Songsasen *et al.* (2017). In brief, ovaries were collected from 40 cats (age, 6 mo – 3 yr) undergoing routine ovariohysterectomy at local veterinary clinics. Gonads were transported to the laboratory within 1 to 5 h post-excision at 4°C while immersed in Leibovitz's L-15 medium. Ovaries were washed twice in Collection Medium (Minimum Essential Medium (MEM) containing 2 mM L-glutamine, 20 mM Hepes, 40 IU/ml penicillin G sodium, 40 µg/ml streptomycin sulfate, and 3 mg/ml bovine serum albumin) before processing for follicle collection.

2.2 Isolation and selection of secondary follicles

Secondary stage follicles (100 – 200 µm in diameter) were mechanically recovered from ovarian cortex as described by Jewgenow and Goritz (1995) and within the Collection Medium. In brief, this involved mechanical dissection of cat ovaries using a cell dissection sieve and the cell suspension passed through a 100 µm cell strainer. The latter was flushed with Collection medium to recover all preantral follicles. Isolated secondary follicles with normal morphology (i.e., round or oval in shape, surrounded by 2 – 3 compact layers of granulosa cells with an intact basement membrane (Jewgenow and Goritz, 1995)) were selected under a stereomicroscope (Nikon SMZ-2T, Tokyo, Japan). Each was evaluated for viability by exposure to 50 µg/ml neutral red stain for 20 min at 37°C (Wongbandue *et al.* 2013). Follicles containing both a neutral red positive oocyte and granulosa cells were classified as viable (Fig. 1) (Repetto *et al.* 2008).

2.3 Assessment of follicle/oocyte growth

Diameters of each follicle and enclosed oocyte were measured at the onset of in vitro culture (Day 0) and on Days 3, 6, 9, 12, and 14 using an inverted microscope (Leitz DMIL, Leica Microsystem, Buffalo Grove, Illinois, USA) with an ocular micrometer. Each follicle was sized from the outer layer of somatic cells, with the measurements including the widest diameter and perpendicular width to the initial assessment. The mean of these two metrics was

calculated and reported as diameter. Oocyte size, excluding the zona pellucida, was evaluated using the same method. Relative growth over time was calculated by the value obtained at each culture assessment divided by that recorded on Day 0. At the end of incubation (Day 14), the presence or absence of an antrum (a fluid-filled cavity) was determined, and follicle viability was again assessed by neutral red staining (as above).

2.4 In vitro culture of secondary follicles

Only morphologically-normal and neutral red positive follicles were encapsulated in 0.5% alginate hydrogel (Pronova UP MVG; Novamatrix, Sandvika, Norway) using described methods from our laboratory (Songsasen *et al.* 2017). Each alginate-enclosed follicle was incubated in 500 µl of basic culture medium (MEM with 2 mM L-glutamine, 40 IU/ml penicillin G sodium, 40 µg/ml streptomycin sulfate, 3 mg/ml BSA, 1.9 µg/ml human transferrin, and 5 µg/ml sodium selenite) supplemented with 1 µg/ml FSH (Folltropin-V; Bioniche Animal Health, Belleville, Ontario, Canada) containing 0 (Control), 1, or 10 µg/ml insulin. All follicles in these three cohorts were incubated in a humidified atmosphere of 5% CO_2 in air for 14 d. Half of the culture medium was changed every 72 h throughout the experimental interval with fresh medium supplemented with insulin (0, 1, or 10 µg/ml according to treatment group).

2.5 RNA extraction

Total RNA was extracted from a group of 20 follicles at the onset of in vitro culture and after 6 or 12 d of incubation (3 replicates/treatment/culture period) using RNAeasy plus mini-kit (Qiagen, Valencia, California, USA) following the manufacturer's protocol. Extracted RNA was treated by a RapidOut DNA removal kit (Thermo-Scientific, Carlsbad, California, USA) to avoid genomic DNA contamination. Quantity of extracted RNA was assessed using a fluorometer (Qubit™ 3.0, Life Technologies, Thermo-Scientific, Carlsbad, California, USA) by the RNA Spike-in method (Li *et al.* 2015).

2.6 Quantitative RT-PCR (qRT-PCR)

Complementary DNA was synthesized from mRNA (2.4 ng/sample) using a Transcriptor High Fidelity cDNA synthesis kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Products were stored at -20□C until qPCR analysis. Sequences of gene-specific primers were referenced from Songsasen *et al.* (2017), except *Aqp9* primers that were designed by the Beacon Designer Software package (Premier Biosoft, Palo Alto, California, USA). Primer sequences are described in Table 1. Relative expression of all genes was normalized to the endogenous control gene *β-actin* that has been shown to be more stable than glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) in RefFinder software (Xie *et al.* 2012). With the exception of *Aqp7*, each PCR reaction (total volume, 20 µl) consisted of 2 µl cDNA and 18 µl of a reaction mixture containing 10 µl of FastStart Essential DNA green master (Roche, Basel, Switzerland), 1 µl each of 10 µM forward and reverse primers, and 6 µl of nuclease-free water. For *Aqp7*, 5 µM of forward and reverse primers were used. Reactions were performed with the following settings: 95°C for 10 min followed by 45 cycles at 95°C for 10 sec, specific annealing temperature (Table 1) for 30 sec, and 72°C for 10 sec. All amplifications were performed in triplicate using LightCycler® 96 (Roche, Basel, Switzerland). Reactions without cDNA were performed in parallel as a negative control. Primer efficiency was assessed in each gene by serial dilution of DNA. Calculation of mRNA expression levels was performed by the comparative Ct method using the amplification efficiency of each gene as a correction factor. Day 0 (non-cultured) follicles were used as a control group for calculations of relative quantitation. Data were reported as n-times difference in relation to the fresh, non-cultured group (Livak and Schmittgen 2001). Data were calculated using Microsoft Excel 2016 software.

2.7 Assessment of follicle morphology

To determine follicular morphology, follicles were processed histologically, as previously described (West-Farrell *et al.* 2009). Briefly, alginate-enclosed follicles at the end of culture (Day 14) were placed in a calcium chloride solution (50 mM $CaCl_2$, 140 mM NaCl) for 2 min followed by fixation in 4% paraformaldehyde, 0.1 M sodium cacodylate, 0.1 M sucrose, and

10 mM calcium chloride for 4 h (4°C). Each fixed follicle then was stained with 0.5% alcian blue and dehydrated through a graded series of ethanol solutions (70 – 100%) followed by embedding in a paraffin block. Serial sections (5 µm) were cut, stained with hematoxylin-eosin, and evaluated by light microscopy (Olympus BX40; Olympus America, Inc.; 400x) for determining the morphology of follicle structure, the presence of theca cells and antral formation.

2.8 Hormone assays

Conditioned medium was collected on Day 12 and then stored at -80°C until shipped to the Endocrine Technologies Support Core (ETSC) at the Oregon National Primate Research Center (Beaverton, Oregon, USA) for analysis of total concentrations of E2, P4, and A4 measured using ultra-high performance liquid chromatography-heated electrospray ionization-tandem triple quadrupole mass spectrometry (LC-MS/MS) on a Shimadzu Nexera-LCMS-8050 instrument (Kyoto, Japan). For sample preparation, 150 µl of culture medium was mixed with 100 µl ultrapure water (Honeywell B&J, Muskegon, Michigan, USA) containing 3.8 ng/ml 17β-oestradiol-D_5, 1.6 ng/ml progesterone-D_9, and 0.8 ng/ml androstene-3,17-dione-2,3,4-C_3 isotopic standards (Cerilliant, Round Rock, Texas, USA) and added to a 400 µl SLE+ extraction plate (Biotage, Charlotte, North Carolina, USA). Steroids were eluted with 900 µl dichloromethane (Merck KGaA, Darmstadt, Germany), dried with forced air, and reconstituted in 50 µl of 25% (v:v) methanol:ultrapure water. Quality control (QC) pools of normal rhesus macaque serum spiked with E4, P4, and A4 standards at concentrations of 1 to 10 ng/ml were also analyzed. QCs were subjected to the same SLE+ extraction procedure with four replicates in each assay. For calibration curves, blank culture medium was spiked with E2, P4, and A4 standards in methanol and diluted serially to final concentrations from 0.015 to 15 ng/ml in a 12-point curve. Spiked standards were then subjected to the SLE+ extraction procedure. After the reconstitution step, samples, standards, and QCs were subjected to LC-MS/MS analysis. Using a Shimadzu SIL-30CAMP autosampler, 25 µl of each sample were injected onto a Raptor 2.7 µm

Biphenyl 50 mm X 2.1 mm column (Restek, Bellefonte, Pennsylvania, USA). The mobile phase consisted of 0.15 mM ammonium fluoride in water (A) and methanol (B) with a flow rate of 0.25 ml/min. Using a Shimadzu Nexera LC-30AD system (LC), gradient elution started at 70% methanol and increased to 100% methanol over 5 min. After chromatography, the column was re-equilibrated back to 70% methanol for 2.75 min for a total of 7.75 min per sample. E2, P4, and A4 had retention times of 1.53, 4.48, and 3.52 min, respectively. E2 was detected in negative ion mode with P4 and A4 detected in positive ion mode with multiple reaction monitoring (MRM) using a Shimadzu LCMS-8050 tandem triple-quadrupole MS with heated electrospray ionization (ESI). All MS parameters were optimized for maximum sensitivity while maintaining analytical robustness prior to sample analysis. The interface temperature was 300°C, the desolvation line temperature was 150°C, and the heat block temperature was 500°C. The dynamic range for the E2, P4, and A4 standard curves was 0.015 to 15 ng/ml. Data processing and analysis were performed using LabSolutions Software, V5.72 (Shimadzu, Japan). Intra-assay variations for E2 were 1.54% and 0.25% for replicate 1 and 2, respectively. The respective values for P4 and A4 were 14.8% and 1.6% (P4) and 16.1% and 1.6% (A4). Because all samples were analyzed using a single assay, no specific inter-assay variation was calculated. Overall inter-assay variation for these three assay systems within the ETSC is less than 15%.

2.9 Experimental design

The design was to determine the impact of insulin supplementation on in vitro follicle growth and survival, mRNA expression of gene regulating steroidogenesis and water transport and steroidogenesis. Cat secondary follicles (n = 646) were isolated from ovarian cortices and cultured in 0 (Control), 1, or 10 µg/ml insulin for 14 d (Day 0 = culture onset). Follicle/oocyte viability (based on neutral red staining), diameter, and antrum formation were assessed every 72 h and at the end of incubation (Day 14). mRNA expression of steroidogenic (*Cyp17a1*, *Cyp19a1*, *Star*) and water transport (*Aqp1*, *Aqp3*, *Aqp7*, *Aqp9*) genes were evaluated on Days 0, 6, and 12 and E2, P4, and A4 concentrations on Day 12.

2.10 Statistical analysis

All data were tested for normality using the Shapiro Wilk normality test and variance homogeneity using the Barlett's test. Follicle and oocyte growth, absence or presence of an antrum cavity, and follicle viability data were normally distributed with equal variance. Comparisons of mean percentages in follicle and oocyte growth within treatment were analyzed using a repeated measure ANOVA followed by a Tukey's multiple comparison test. Mean percentages in follicle and oocyte growth, absence or presence of antrum cavity, and follicle viability among treatment groups were compared using a one way ANOVA followed by a Tukey's multiple comparison test. Differences in mRNA expression were evaluated using a Kruskal-Wallis test with a Dunn's multiple comparison test. All statistical analyses were performed using Graphpad Prism version 5.0 for Windows (GraphPad Software, La Jolla, California, USA). Differences with a value of $P < 0.05$ were considered statistically significant.

3. Results

Insulin enhanced ($P < 0.05$) viability of cultured follicles. Specifically, more neutral red positive follicles were observed on Day 14 after the high (86%) compared to the Control (38.1%) or low (59.8%) insulin treatment. Follicles exposed to insulin (1 or 10 μg/ml) increased in diameter as in vitro culture progressed (Fig. 2a), whereas those cultured without the hormone remained the same size through the 14 d incubation. Insulin supplementation dose-dependently increased ($P < 0.05$) the proportion of follicles forming an antrum. Seventy-eight percent of follicles exposed to high insulin developed a distinctive antral cavity compared to only 3.3% and 26.9% of those in no and low insulin groups, respectively (Fig. 3a-c, Fig. 4). Histological analysis revealed presence of theca cell layers on follicles (on Day 14) incubated in high insulin supplementation (Fig. 4d). Although sustaining oocyte viability (based on neutral red staining), supplemental insulin had no influence on oocyte growth; egg

diameter on Day 14 in all groups was no different ($P > 0.05$) from that on Day 0 at culture onset (Fig. 2b).

Our evaluation revealed that insulin influenced temporal mRNA expression of steroidogenic genes across treatment groups, especially on Day 6. Specifically, *Cyp17a1* expression was higher ($P < 0.05$) in the low compared to high insulin treatment (with the 0 dosage producing an intermediate, non-significant difference) (Fig. 5a). Similar to what occurred on Day 6, mRNA levels on Day 12 were increased ($P < 0.01$) by exposure to low compared to high insulin concentration. Although *Cyp19a1* mRNA level was similar ($P > 0.05$) among Control and treatment groups (Fig. 5b), *Star* expression in follicles cultured for 6 d in low insulin was higher ($P < 0.01$) than the no insulin counterpart. There was no evidence of *Star* mRNA expression by Day 12 in the presence of high insulin (Fig. 5c).

In contrast to what occurred for steroidogenic genes, expression of *Aqp3* and *Aqp7* was unaffected ($P > 0.05$) by treatment (Fig. 6). However, *Aqp1* expression was up-regulated ($P < 0.01$) on Day 6 in the low compared to high insulin condition. Although insulin had no effect on *Aqp9* expression on Day 6, transcript level of this gene was influenced ($P < 0.05$) by high insulin supplementation with down-regulation on Day 12 compared to the Control (Fig. 6).

Analysis of spent medium collected on Day 12 revealed that E2 and A4 concentrations were not different ($P > 0.05$) between the Control and either the low or high insulin treatment groups (Fig. 7a&c). However, low insulin increased progesterone concentration compared to no ($P < 0.01$) or high ($P < 0.001$) insulin counterparts (Fig. 7b).

4. Discussion

Our study in the cat model examined secondary ovarian follicles cultured in vitro and added to the informational database by making three main discoveries. First, we observed a dose-dependent response of insulin on antrum formation, growth, follicle survival and theca cell

differentiation. Secondly, insulin assisted in sustaining oocyte viability without influencing actual growth and ultimate size of the egg. Lastly, insulin affected temporal expression of specific genes regulating steroidogenesis and water transport, while having an additional effect (at low dosage) on enhanced progesterone production in vitro. Collectively, these findings revealed that insulin appeared to be a key endocrine factor in successful folliculogenesis in the domestic cat.

Earlier studies have demonstrated the important roles of insulin in ovarian follicle activation and growth both in vivo and in vitro (Selvaraju *et al.* 2003; Rossetto *et al.* 2016). In the mouse, insulin promotes primordial follicular assembly and activation by stimulating the PI3K/Akt signaling pathway (Zhang *et al.* 2010). Insulin also enhances cell proliferation and differentiation of incubated goat preantral follicles (Chaves *et al.* 2012) and facilitates FSH-dependent steroidogenesis in cultured pig granulosa cells (May *et al.* 1980). Insulin also is known to play a key role in in vitro activation of primordial follicles enclosed within incubated ovarian cortices in the rat (Kezele *et al.* 2002), goat (Chaves *et al.* 2011), cow (Fortune *et al.* 2010), and human (Louhio *et al.* 2000). Supplemental insulin also has been found to enhanced survival of secondary stage, cultured follicles of the dog (Serafim *et al.* 2013) and goat (Ferreira *et al.* 2016), in both cases by promoting formation of the follicular antrum. Goat oocytes within such insulin-exposed follicles are better able to resume meiosis and ultimately achieve metaphase II (Silva *et al.* 2017). Our findings confirmed that impact of insulin on incubated, immature follicles appeared conserved across species. Cat secondary follicles reacted similarly to those of the dog (another carnivore; Serafim *et al.* 2013), goat (Chaves *et al.* 2012; Ferreira *et al.* 2016), and human (Louhio *et al.* 2000). More specifically, supplemental insulin sustained follicular and oocyte viability while increasing follicle size by 30% above control values. There also was an affirmation on influence of this hormone (especially at high dosage) to enhance antrum formation and enlargement, likely by stimulating theca cell differentiation that, in turn, facilitated flow of extrafollicular fluid into the antral cavity. This speculation is supported by earlier studies of the mouse and human where

either insulin or insulin-like-growth factor I is known to promote ovarian follicle growth by inducing theca cell differentiation and proliferation (Young and McNeilly 2010), probably by regulating cell metabolism through signal transduction pathways, such as Ras-MAPK and PI3K-Akt (Avruch 1998).

Insulin dose-dependency appeared focused strictly on oocyte survival (based on neutral red staining) with no influence on the growth of gamete itself. Oocyte diameter at onset of culture in our study (~65 μm) was consistent with that of eggs derived from in vivo-developed, secondary cat follicles (Reynaud *et al.* 2009). However, our cultured follicles on Day 14 produced oocytes (also ~65 μm), even in the presence of high insulin, that were far smaller in size than in vivo-derived counterparts from late secondary (~86 μm) or early antral (~108 μm) stages (Reynaud *et al.* 2009). This finding was contrary to that reported in the goat where supplementing preantral follicles with insulin increased both diameter and ability to resume meiosis of the resident oocyte over an 18 d incubation (Ferreira *et al.* 2016). The differences between what was observed in the goat and cat models probably was related to the stage of the donor follicle. For example, it is known that the diameter of oocytes within primordial goat follicles is unaffected in vitro by exposure to insulin (Chaves *et al.* 2011). Therefore, we speculate that oocytes from more advanced follicles are likely to be more responsive to an insulin stimulus, a possibility that deserves further study. Alternatively, there simply may be species variabilities in sensitivity at the oocyte level to this peptide hormone, a possibility that will be understood as the influence of insulin is explored in other animal models.

One mechanism whereby insulin stimulates follicle growth is by up-regulating genes that control steroidogenesis, including *Star* (facilitates transport of cholesterol required for steroidogenesis), *Cyp11a1* (converts cholesterol to pregnenolone), and *Cyp17a1* (converts pregnenolone to dehydroepiandrosterone, DHEA) (Young and McNeilly 2010). In the present study, insulin, especially at a low concentration increased mRNA expression of *Star* and *Cyp17a1* after 6 d of culture. The up-regulation of *Star* during low insulin exposure was

confirmed physiologically by a parallel increase in P4 concentration over the entire incubation period. However, the up-regulation of *Cyp17a1* in low insulin failed to translate into increased A4 concentration in culture. This inability of insulin supplementation to influence A4 production suggested that the cat may be similar to the human where the P450c17 enzyme is unable to efficiently convert 17β-hydroxyprogesterone to A4, a finding in contrast to that observed in cattle and rodents (Miller *et al.* 2006). Despite the stimulating effect of low insulin exposure on *Cyp17a1*, the higher dosage had no influence on this gene and, furthermore, diminished *Star* expression by the end of culture. There is early evidence in the cow that a lower concentration of insulin supplementation enhances E2 and P4 production in granulosa cell cultures, whereas increased insulin has no impact (Spicer *et al.* 1993). In the present study, the undetectable level of *Star* transcript and low P4 concentration at Day 12 in the presence of high insulin suggested that persistent elevations of this steroidogenic transcript and P4 production are not a prerequisite for antrum formation. In fact, up-regulation of *Star* mRNA and P4 production has been shown to be associated with poor antrum formation in incubated mouse preantral follicles (West-Farrell *et al.* 2009).

In the present study, we demonstrated for the first time that insulin influenced temporal expression of water transport genes within the incubated mammalian follicle. We observed an absence of *Aqp7* by Day 6 when cat follicles were exposed to high insulin. However, the transcript was detected at the level similar to the low (or control) insulin counterparts on Day 12. It is known that *Aqp7* is located in sheep follicular cells (Sales *et al.* 2015; Lee *et al.* 2016). Therefore, the increase in this transcript in high insulin treatment was likely the result of granulosa cell proliferation associated with insulin supplementation (Jewgenow and Göritz 1995) rather than the direct impact of this hormone on the water transport gene.

Our finding that high insulin suppressed transcription of *Aqp9* was similar to previous observations made for human fetal membrane (Bouvier *et al.* 2015) and placental tissue (Castro Parodi *et al.* 2011). These particular in vitro studies determined that insulin increases phosphorylation of Akt proteins, a process that then impairs the intracellular phosphorylation

pathway and down-regulates *Aqp9* expression. However, it also is noteworthy that immuno-localization of this water transport gene is associated with formation of the follicular antrum in sheep (Sales *et al.* 2015). Thus, future studies should examine the impact of insulin on protein expression of AQP9 in incubated cat follicles to verify its inter-relationship to insulin in antral formation. Finally, for *Aqp1*, the transcript level increased only in the presence of low insulin and only on Day 6, an effect lost by Day 12. In the human, mRNA and protein expression of AQP1 is known to increase during the late follicular phase and post-ovulation, suggesting that this gene is likely most associated with the peri-ovulatory period, ovulation, and corpus luteum formation (Thoroddsen *et al.* 2011). Therefore, the role of AQP1 in the cat may well be the same (or quite similar to the human), coming into functional play at the end of folliculogenesis.

In summary, this study helped provide another level of understanding about control of folliculogenesis, including how to advance ovarian follicle (and resident oocyte) development in an artificial microenvironment. Our findings in the cat model add credence to observations made in eight other mammalian species that insulin is a key regulator of growth and differentiation of secondary follicles, and dose dependently. It appeared that insulin's stimulating mechanism was through ensuring appropriate temporal expression of *Cyp17a1* and *Star* as well as progesterone production. Earlier investigations (Edashige *et al.* 2000; McConnell *et al.* 2002; Skowronska *et al.* 2009) have suggested that AQPs, especially *Aqp1*, *Aqp3*, *Aqp7*, *Aqp8*, and *Aqp9*, may play a role in antral cavity formation. But such efforts have focused on examining AQP localization within the ovary (Edashige *et al.* 2000; Lee *et al.* 2016; Skowronska *et al.* 2009; Thoroddsen *et al.* 2011). Ours was the first to examine these water transport genes in the follicle itself, demonstrating yet another new mechanism whereby insulin has influence through temporal mRNA expression of two (*Aqp1* and *Aqp9*) of these five transcripts. This finding also provided encouragement for the need to identify other key regulators of AQPs expression during folliculogenesis as antral cavity expansion is fundamental to follicular maturation. Likely candidates include ovarian steroids, cyclic

adenosine monophosphate, forskolin, and prostaglandins (Hua *et al.* 2013). We are confident that building upon this collective knowledge will lead to the ability to create an in vitro system that permits appropriate antral cavity expansion to allow producing oocytes that can mature, fertilize, and develop into a viable embryo.

Acknowledgement

The authors thank veterinary hospitals in Front Royal, Stephens City, and Harrisonburg, Virginia, USA for providing cat ovaries and the Endocrine Technologies Support Core (ETSC) at the Oregon National Primate Research Center (ONPRC). ETSC was supported by National Institutes of Health Grant P51 OD011092 awarded to ONPRC. This research was supported by the National Institute of Health (Grant R56OD018304), Smithsonian Institution, and a gift from Dr. Clint and Missy Kelly.

Conflicts of interest

The author declares that there is no conflict of interest.

References

Asai, M., Higuchi, S., Kubota, M., Iguchi, K., Usui, S., and Hirano, K. (2006) Regulators for blood glucose level affect gene expression of aquaporin 3. *Biol. Pharm. Bull.* **29**(5), 991-996.

Avruch, J. (1998) Insulin signal transduction through protein kinase cascades. *Mol. Cell. Biochem.* **182**(1-2), 31-48.

Barrett, S.L., and Woodruff, T.K. (2010) Gamete preservation. *Cancer Treat. Res.* **156**, 25-39.

Bouvier, D., Rouzaire, M., Marceau, G., Prat, C., Pereira, B., Lemarié, R., Deruelle, P., Fajardy, I., Gallot, D., Blanchon, L., Vambergue, A., and Sapin, V. (2015) Aquaporins and fetal membranes from diabetic parturient women: expression abnormalities and regulation by insulin. *J. Clin. Endocrinol. Metab.* **100**(10), 1270-1279.

Camacho, P., Fan, H., Liu, Z., and He, J. (2016) Small mammalian animal model of heart disease. *Am. J. Cardiovasc. Dis.* **6**(3), 70-80.

Castro Parodi, M., Farina, M., Dietrich, V., Abán, C., Szpilbarg, N., Zotta, E., and Damiano, A.E. (2011) Evidence for insulin-mediated control of AQP9 expression in human placenta. *Placenta* **32**(12), 1050-1056.

Chaves, R., Alves, A., Faustino, L., Oliveira, K., Campello, C., Lopes, C., Báo, S., and Figueiredo, J. (2011) How the concentration of insulin affects the development of preantral follicles in goats. *Cell Tissue Res.* **346**(3), 451-456.

Chaves, R.N., Duarte, A.B., Rodrigues, G.Q., Celestino, J.J., Silva, G.M., Lopes, C.A., Almeida, A.P., Donato, M.A., Peixoto, C.A., Moura, A.A., Lobo, C.H., Locatelli, Y., Mermillod, P., Campello, C.C., and Figueiredo, J.R. (2012) The effects of insulin and follicle-simulating hormone (FSH) during in vitro development of ovarian goat preantral follicles and the relative mRNA expression for insulin and FSH receptors and cytochrome P450 aromatase in cultured follicles. *Biol. Reprod.* **87**(3), 1-11.

Comizzoli, P., Crosier, A.E., Songsasen, N., Gunther, M.S., Howard, J.G., and Wildt, D.E. (2009) Advances in reproductive science for wild carnivore conservation. *Reprod. Domest. Anim.* **44** (Suppl 2), 47-52.

Duleba, A.J., Spaczynski, R.Z., Olive, D.L., and Behrman, H.R. (1997) Effects of insulin and insulin-like growth factors on proliferation of rat ovarian theca-interstitial cells. *Biol. Reprod.* **56**(4), 891-897.

Edashige, K., Sakamoto, M., and Kasai, M. (2000) Expression of mRNAs of the aquaporin family in mouse oocytes and embryos. *Cryobiology* **40**(2), 171-175.

Eppig, J.J., and O'Brien, M.J. (1996) Development in vitro of mouse oocytes from primordial follicles. *Biol. Reprod.* **54**(1), 197-207.

Ferreira, A., Maside, C., Sá, N., Guerreiro, D., Correia, H., Leiva-Revilla, J., Lobo, C., Araújo, V., Apgar, G., and Brandão, F. (2016) Balance of insulin and FSH concentrations improves the in vitro development of isolated goat preantral follicles in medium containing GH. *Anim. Reprod. Sci.* **165**, 1-10.

Fujihara, M., Comizzoli, P., Wildt, D.E., and Songsasen, N. (2012) Cat and dog primordial follicles enclosed in ovarian cortex sustain viability after in vitro culture on agarose gel in a protein-free medium. *Reprod. Domest. Anim.* **47**(6), 102-108.

Fujihara, M., Comizzoli, P., Keefer, C.L., Wildt, D.E., and Songsasen, N. (2014) Epidermal growth factor (EGF) sustains in vitro primordial follicle viability by enhancing stromal cell proliferation via MAPK and PI3K pathways in the prepubertal, but not adult, cat ovary. *Biol. Reprod.* **90**(4), 86,1–10.

Gupta, P.S.P., Ramesh, H.S., Manjunatha, B.M., Nandi, S., and Ravindra, J.P. (2008) Production of buffalo embryos using oocytes from in vitro grown preantral follicles. *Zygote* **16**(1), 57-63.

Higuchi, S., Kubota, M., Iguchi, K., Usui, S., Kiho, T., and Hirano, K. (2007) Transcriptional regulation of aquaporin 3 by insulin. *J. Cell. Biochem.* **102**(4), 1051-1058.

Hovatta, O. (2004) Cryopreservation and culture of human ovarian cortical tissue containing early follicles. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **113**(Suppl 1), S50-54.

Hua, Y., Jiang, W., Zhang, W., Shen, Q., Chen, M., and Zhu, X. (2013) Expression and significance of aquaporins during pregnancy. *Front. Biosci.* **18**, 1373-1383.

Huang, H.F., He, R.H., Sun, C.C., Zhang, Y., Meng, Q.X., and Ma, Y.Y. (2006) Function of aquaporins in female and male reproductive systems. *Hum. Reprod. Update* **12**(6), 785-795.

Hunter, M., Robinson, R., Mann, G., and Webb, R. (2004) Endocrine and paracrine control of follicular development and ovulation rate in farm species. *Anim. Reprod. Sci.* **82-83**, 461-477.

Jewgenow, K., and Göritz, F. (1995) The recovery of preantral follicles from ovaries of domestic cats and their characterisation before and after culture. *Anim. Reprod. Sci.* **39**(4), 285-297.

Knight, P.G., and Glister, C. (2003) Local roles of TGF-β superfamily members in the control of ovarian follicle development. *Anim. Reprod. Sci.* **78**(3), 165-183.

Lee, H.J., Jee, B.C., Kim, S.K., Kim, H., Lee, J.R., Suh, C.S., and Kim, S.H. (2016) Expressions of aquaporin family in human luteinized granulosa cells and their correlations with IVF outcomes. *Hum. Reprod.* **31**(4), 822-831.

Li, X., Ben-Dov, I.Z., Mauro, M., and Williams, Z. (2015) Lowering the quantification limit of the Qubit TM RNA HS Assay using RNA spike-in. *BMC Mol. Biol.* **16**, 9

Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods* **25**(4), 402-408.

Louchami, K., Best, L., Brown, P., Virreira, M., Hupkens, E., Perret, J., Devuyst, O., Uchida, S., Delporte, C., and Malaisse, W.J. (2012) A new role for aquaporin 7 in insulin secretion. *Cell. Physiol. Biochem.* **29**(1-2), 65-74.

Louhio, H., Hovatta, O., Sjöberg, J., and Tuuri, T. (2000) The effects of insulin, and insulin-like growth factors I and II on human ovarian follicles in long-term culture. *Mol. Hum. Reprod.* **6**(8), 694-698.

Luz, V.B., Araujo, V.R., Duarte, A.B., Celestino, J.J., Silva, T.F., Magalhaes-Padilha, D.M., Chaves, R.N., Brito, I.R., Almeida, A.P., Campello, C.C., Feltrin, C., Bertolini, M., Santos, R.R., and Figueiredo, J.R. (2012) Eight-cell parthenotes originated from in vitro grown sheep preantral follicles. *Reprod. Sci.* **19**(11), 1219-1225.

Magalhaes, D.M., Duarte, A.B., Araujo, V.R., Brito, I.R., Soares, T.G., Lima, I.M., Lopes, C.A., Campello, C.C., Rodrigues, A.P., and Figueiredo, J.R. (2011) In vitro production of a

caprine embryo from a preantral follicle cultured in media supplemented with growth hormone. *Theriogenology* **75**(1), 182-188.

May, J.V., McCarty, K., Jr., Reichert, L.E., Jr., and Schomberg, D.W. (1980) Follicle-stimulating hormone-mediated induction of functional luteinizing hormone/human chorionic gonadotropin receptors during monolayer culture of porcine granulosa cells. *Endocrinology* **107**(4), 1041-1049.

McConnell, N.A., Yunus, R.S., Gross, S.A., Bost, K.L., Clemens, M.G., and Francis M. Hughes, J. (2002) Water permeability of an ovarian antral follicle is predominantly transcellular and mediated by aquaporins. *Endocrinology* **143**(8), 2905-2912.

Moulavi, F., Hosseini, S.M., Tanhaie-Vash, N., Ostadhosseini, S., Hosseini, S.H., Hajinasrollah, M., Asghari, M.H., Gourabi, H., Shahverdi, A., Vosough, A.D., and Nasr-Esfahani, M.H. Interspecies somatic cell nuclear transfer in Asiatic cheetah using nuclei derived from post-mortem frozen tissue in absence of cryo-protectant and in vitro matured domestic cat oocytes. *Theriogenology* **1**(90), 197-203.

Narfström, K., Deckman, K.H., and Menotti-Raymond, M. (2013) Cats: a gold mine for opthalmology. *Annu. Rev. Anim. Biosci.* **1**, 157-177

Pelican, K.M., Wildt, D.E., and Howard, J.G. (2006) GnRH agonist Lupron (R) (leuprolide acetate) pre-treatments prevent ovulation in response to gonadotropin stimulation in the clouded leopard (*Neofelis nebulosa*). *Theriogenology* **66**(6-7), 1768-1777.

Picton, H.M., Harris, S.E., Muruvi, W., and Chambers, E.L. (2008) The in vitro growth and maturation of follicles. *Reproduction* **136**(6), 703-715.

Quesnel, H. (1999) Localization of binding sites for IGF-I, insulin and GH in the sow ovary. *J. Endocrinol.* **163**(2), 363-372.

Repetto, G., del Peso, A., and Zurita, J.L. (2008) Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat. Protc.* **3**(7), 1125-1131.

Reynaud, K., Gicquel, C., Thoumire, S., Chebrout, M., Ficheux, C., Bestandji, M., and Chastant-Maillard, S. (2009) Folliculogenesis and morphometry of oocyte and follicle growth in the feline ovary. *Reprod. Domest. Anim.* **44**(2), 174-179.

Rossetto, R., Saraiva, M., Bernuci, M., Silva, G., Brito, I., Alves, A., Magalhães-Padilha, D., Báo, S., Campello, C., and Rodrigues, A. (2016) Impact of insulin concentration and mode of FSH addition on the in vitro survival and development of isolated bovine preantral follicles. *Theriogenology* **86**(4), 1137-1145.

Sales, A., Duarte, A., Rodrigues, G., Lima, L., Silva, G., Carvalho, A., Brito, I., da Maranguape, R., Lobo, C., and Aragão, J. (2015) Steady-state level of messenger RNA and immunolocalization of aquaporins 3, 7, and 9 during in vitro growth of ovine preantral follicles. *Theriogenology* **84**(1), 1-10.

Selvaraju, S., Agarwal, S., Karche, S., and Majumdar, A. (2003) Ovarian response, embryo production and hormonal profile in superovulated goats treated with insulin. *Theriogenology* **59**(5), 1459-1468.

Serafim, M.K., Silva, G.M., Duarte, A.B., Araújo, V., Silva, T., Lima, A., Chaves, R., Campello, C., Silva, L., and Figueiredo, J. (2013) High insulin concentrations promote the in vitro growth and viability of canine preantral follicles. *Reprod. Fertil. Dev.* **25**(6), 927-934.

Shimizu, T., Murayama, C., Sudo, N., Kawashima, C., Tetsuka, M., and Miyamoto, A. (2008) Involvement of insulin and growth hormone (GH) during follicular development in the bovine ovary. *Anim. Reprod. Sci.* **106**(1-2), 143-152.

Silva, G., Brito, I., Sales, A., Aguiar, F., Duarte, A., Araújo, V., Vieira, L., Magalhães-Padilha, D., Lima, L., and Alves, B. (2017) In vitro growth and maturation of isolated caprine preantral follicles: Influence of insulin and FSH concentration, culture dish, coculture, and oocyte size on meiotic resumption. *Theriogenology* **90**, 32-41.

Silva, J., and Price, C. (2002) Insulin and IGF-I are necessary for FSH-induced cytochrome P450 aromatase but not cytochrome P450 side-chain cleavage gene expression in oestrogenic bovine granulosa cells in vitro. *J. Endocrinol*. **174**(3), 499-507.

Skowronska, M.T., Kwon, T.H., and Nielsen, S. (2009) Immunolocalization of aquaporin 1, 5, and 9 in the female pig reproductive system. *J. Histochem. Cytochem.* **57**(1), 61-67.

Songsasen, N., Comizzoli, P., Nagashima, J., Fujihara, M., and Wildt, D.E. (2012) The domestic dog and cat as models for understanding the regulation of ovarian follicle development in vitro. *Reprod. Domest. Anim.* **47**(Suppl 6), 13-18.

Songsasen, N., Thongkittidilok, C., Yamamizu, K., Wildt, D., and Comizzoli, P. (2017) Short-term hypertonic exposure enhances in vitro follicle growth and meiotic competence of enclosed oocytes while modestly affecting mRNA expression of aquaporin and steroidogenic genes in the domestic cat model. *Theriogenology* **90**, 228-236.

Spicer, L., Alpizar, E., and Echternkamp, S. (1993) Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and (or) insulin-like growth factor I production in vitro. *J. Anim. Sci.* **71**(5), 1232-1241.

Thoroddsen, A., Dahm-Kähler, P., Lind, A.K., Weijdegård, B., Lindenthal, B., Müller, J., and Brännström, M. (2011) The water permeability channels aquaporins 1–4 are differentially expressed in granulosa and theca cells of the preovulatory follicle during precise stages of human ovulation. *J. Clin. Endocrinol. Metab.* **96**(4), 1021-1028.

Thuwanut, P., Comizzoli, P., Wildt, D.E., Keefer, C.L., and Songsasen, N. (2016) Stem cell factor (SCF) promotes in vitro ovarian follicle development in the domestic cat by upregulating c-kit mRNA expression and stimulating PI3K-Akt pathway. *Reprod. Fertil. Dev.* **29**(7), 1356-1368.

Troyer, J.L., Roelke, M.E., Jespersen, J.M., Baggett, N., Buckley-Beason, V., MacNulty, D., Craft, M., Packer, C., Pecon-Slattery, J., and O'Brien, S.J. (2011) FIV diversity: FIV Ple subtype composition may influence disease outcome in African lions. *Vet. Immunol. Immunopathol.* **143**(3-4), 338-346.

West-Farrell, E.R., Xu, M., Gomberg, M.A., Chow, Y.H., Woodruff, T.K., and Shea, L.D. (2009) The mouse follicle microenvironment regulates antrum formation and steroid production: alterations in gene expression profiles. *Biol. Reprod.* **80**(3), 432-439.

Willis, D., Mason, H., Gilling-Smith, C., and Franks, S. (1996) Modulation by insulin of follicle-stimulating hormone and luteinizing hormone actions in human granulosa cells of normal and polycystic ovaries. *J. Clin. Endocrinol. Metab.* **81**(1), 302-309.

Wongbandue, G., Jewgenow, K., and Chatdarong, K. (2013) Effects of thyroxin (T4) and activin A on in vitro growth of preantral follicles in domestic cats. *Theriogenology* **79**(5), 824-832.

Wu, J., Emery, B.R., and Carrell, D.T. (2001) In vitro growth, maturation, fertilization, and embryonic development of oocytes from porcine preantral follicles. *Biol. Reprod.* **64**(1), 375-381.

Xie, F., Xiao, P., Chen, D., Xu, L., and Zhang, B. (2012) miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol. Biol.* **80**(1), 75-84.

Xu, M., Fazleabas, A.T., Shikanov, A., Jackson, E., Barrett, S.L., Hirshfeld-Cytron, J., Kiesewetter, S.E., Shea, L.D., and Woodruff, T.K. (2011) In vitro oocyte maturation and preantral follicle culture from the luteal-phase baboon ovary produce mature oocytes. *Biol. Reprod*. **84**(4), 689-697.

Xu, M., Kreeger, P.K., Shea, L.D., and Woodruff, T.K. (2006) Tissue-engineered follicles produce live, fertile offspring. *Tissue Eng.* **12**(10), 2739-2746.

593 Xu, M., West-Farrell, E.R., Stouffer, R.L., Shea, L.D., Woodruff, T.K., and Zelinski, M.B.
594 (2009) Encapsulated three-dimensional culture supports development of nonhuman primate
595 secondary follicles. *Biol. Reprod.* **81**(3), 587-594.

596 Young, J., and McNeilly, A.S. (2010) Theca: the forgotten cell of the ovarian follicle.
597 *Reproduction* **140**(4), 489-504.

598

599

600 **Figure Legends**

601 **Fig. 1.** Micrographs of (a) (non-viable, neutral red negative) and (b) (viable, neutral red
602 positive cat follicles at Day 14 of in vitro culture. Bar =150 μm.

603 **Fig. 2.** Diameter (μm); Mean ± SEM of secondary follicle (a) and oocytes (b) cultured for 14
604 d in medium supplemented with 0, 1 or 10 μg/ml insulin. ^{A,B,C,D,E} Superscripts indicate
605 significant difference among culture days within the same treatment, $P < 0.05$. ^{a,b,c} indicate
606 significant difference among treatments within the same culture day, $P < 0.05$.

607 **Fig. 3.** Micrographs of cat follicles cultured in the absence of insulin (a), 1 μg/ml (b), and 10
608 μg/ml (c) insulin. Bar = 150 μm. Black arrow head indicates antral cavity.

609 **Fig. 4.** Histological images of non-cultured follicle (a), cultured follicles on Day 14 with insulin
610 supplementation at 0 (b), 1 (c) and 10 (d) μg/ml. Bar = 100 μm. O, oocyte; GC, granulosa
611 cells; a, antrum; black arrow head, theca cells.

612 **Fig. 5.** Relative expression (based on the fresh, non-cultured control) of gene regulating
613 steroidogenesis (*Cyp17a1* (a), *Cyp19a1* (b) and *Star* (c) in follicles at Day 6 and 12 of culture
614 in medium supplemented with insulin (0, 1 or 10 μg/ml). Bars show mean ± SEM. Asterisks
615 indicate significant levels. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Fig. 6. Relative expression (based on the fresh, non-cultured control) of gene regulating water transport (*Aqp1* (a), *Aqp3* (b), *Aqp7* (c) and *Aqp9* (d) in follicles at Day 6 and 12 of culture in medium supplemented with insulin (0, 1 or 10 μg/ml). Bars show mean ± SEM. Asterisks indicate significant levels. * $P \leq 0.05$, ** $P \leq 0.01$.

Fig. 7. Oestradiol (a), progesterone (b) and androstenedione (c) production at Day 12 of culture by cat follicles cultured in medium supplemented with insulin (0, 1 or 10 μg/ml). Bars show mean ± SEM. Asterisks indicate significant levels. ** $P < 0.01$, *** $P < 0.001$.

Fig. 1

(a) (b)

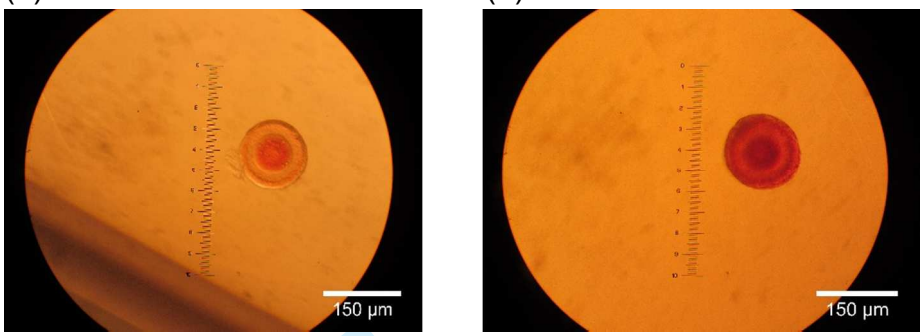

Fig. 2

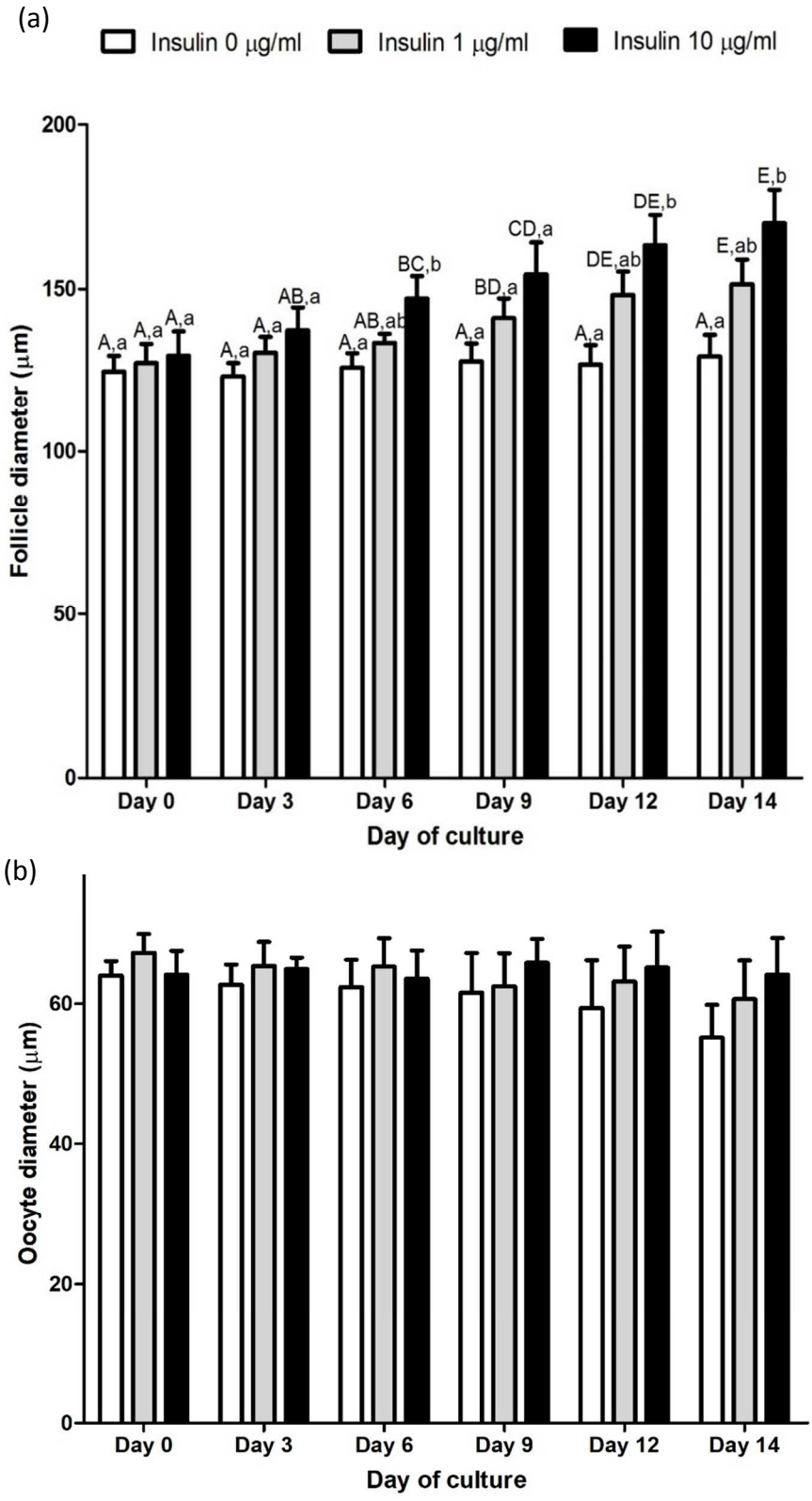
(a)
Insulin 0 μg/ml
Insulin 1 μg/ml
Insulin 10 μg/ml
Follicle diameter (μm)
200
150
100
50
0
A,a
A,a
A,a
A,a
A,a
AB,a
A,a
AB,ab
BC,b
A,a
BD,a
CD,a
A,a
DE,ab
DE,b
A,a
E,ab
E,b
Day 0
Day 3
Day 6
Day 9
Day 12
Day 14
Day of culture
(b)
Oocyte diameter (μm)
60
40
20
0
Day 0
Day 3
Day 6
Day 9
Day 12
Day 14
Day of culture

Fig. 3

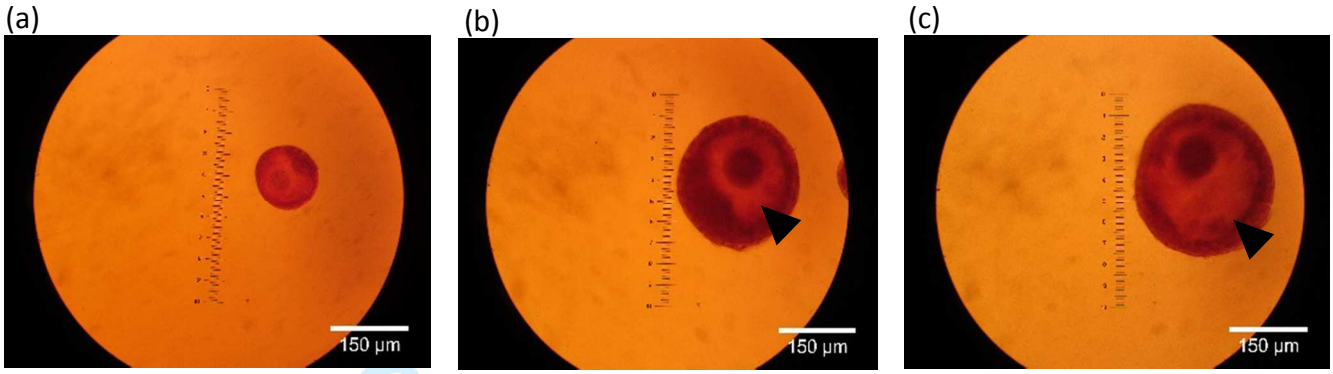

Fig. 4

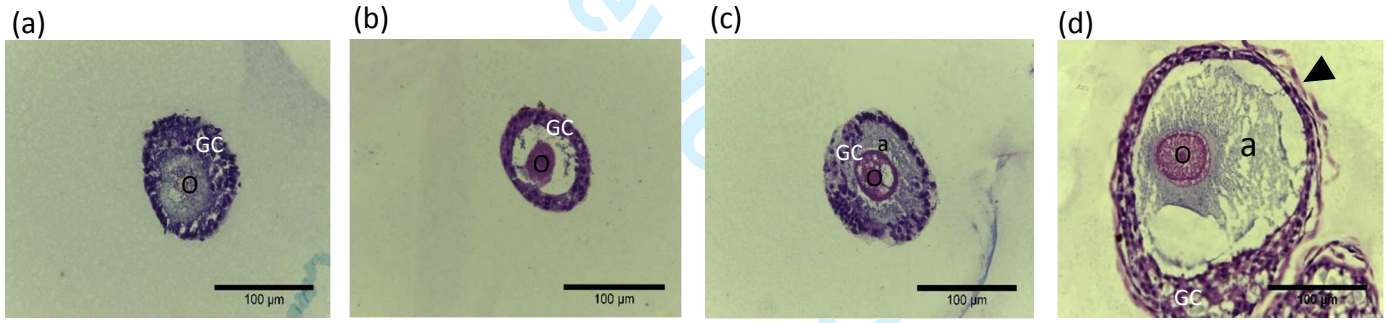

Fig. 5

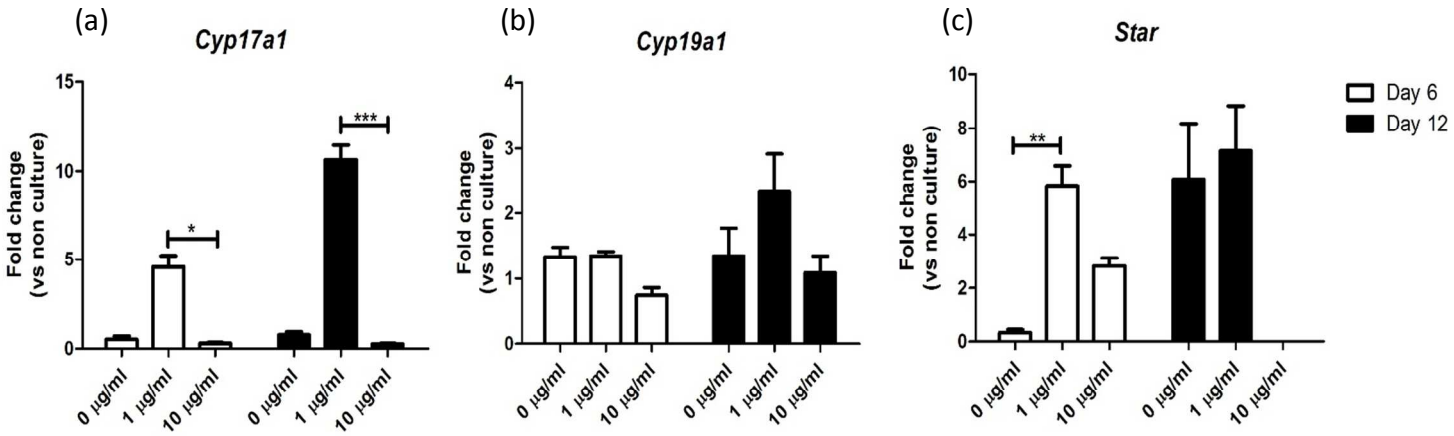

Fig. 6

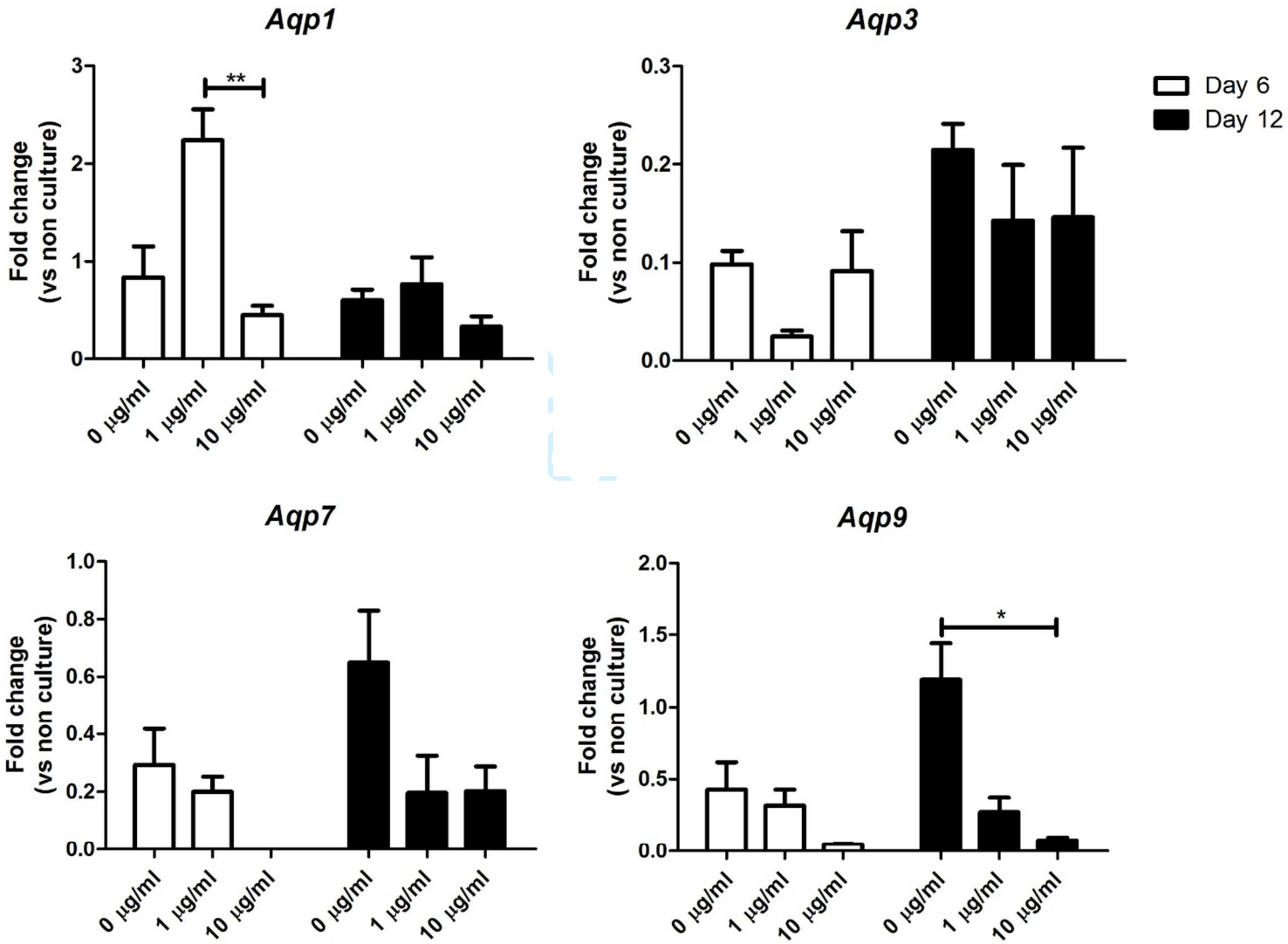

Fig. 7

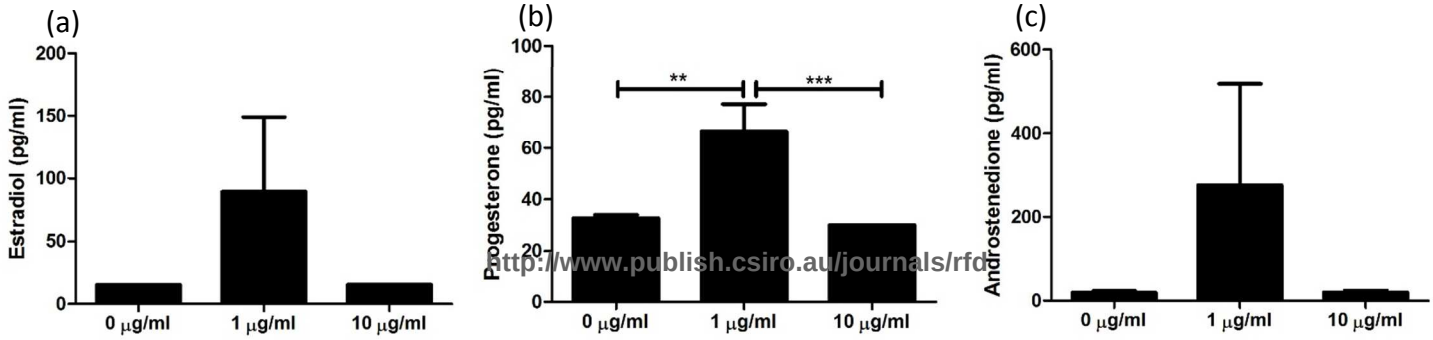

Table 1. List of primers used in this study

Gene	Sense	Sequence 5'-3'	Accession number	Annealing temperature (°C)	Product length (bp)	Primer efficiency
β-actin	Forward	ATCCACGAGACCACCTTC	AB051104.1	57	75	1.077
	Reverse	CACCGTGTTAGCGTAGAG				
Gapdh	Forward	CATCACCATCTTCCAGGA	NM_001009307	57	81	1.009
	Reverse	CCAGTAGACTCCACAACA				
Cyp17a1	Forward	CCGAGATGAGTTGCTGAG	NM_001009371.2	57	105	0.87
	Reverse	GAGTTCATCCTGGCTTGG				
Cyp19a1	Forward	CAATCCTGCTGCTCACTG	GU306147.1	57	84	0.917
	Reverse	CCATGCAATAGCCAGGAC				
Star	Forward	ATGGAAGCGATGGGAGAG	NM_001246196.1	57	90	1.066
	Reverse	CAACTCGTGGGTGATGAC				
Aqp1	Forward	GGCTCGTCAGTGATTACTC	XM_003982907.3	60	103	0.924
	Reverse	CCAGGATGAAGTCGTAGATG				
Aqp3	Forward	ATCTATGCCTTGGCTCAG	XM_011288588.1	59	96	1.056
	Reverse	CTCATTCTTGGCGAAGTC				
Aqp7	Forward	GCATGTCCTGGAAGAAGT	XM_006939211.2	57	78	1.321
	Reverse	AGTAGATGGTGGCAGAAG				
Aqp9	Forward	GTCTTGAAGAGCACCTTAGC	XM_003987104.3	57.5	85	1.038
	Reverse	CAACAGAGCCACATCCAA				