Absence of seasonal changes in \textit{FSHR} gene expression in the cat cumulus–oocyte complex \textit{in vivo} and \textit{in vitro}

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J Howard was deceased

Abstract

Domestic cat oocytes are seasonally sensitive to FSH. Compared with those collected during the breeding season, oocytes from the nonbreeding (NB) season require more FSH during \textit{in vitro} maturation to achieve comparable developmental competence. This study tested the hypothesis that this seasonal variation was due to altered expression of FSH receptors (FSHR) and/or FSH-induced genes. Relative expression levels of \textit{FSHR} mRNA and FSH-enhanced gene estrogen receptor \textit{b} (\textit{ESR2}) were measured by qPCR in whole ovaries and immature cumulus–oocyte complexes (COCs) isolated from cat ovaries during the natural breeding vs NB seasons. Expression levels of FSH-induced genes prostaglandin-endoperoxide synthase 2 (\textit{PTGS2}), early growth response protein-1 (\textit{EGR1}), and epidermal growth factor receptor (\textit{EGFR}) were examined in mature COCs from both seasons that were a) recovered \textit{in vivo} or b) matured \textit{in vitro} with conventional (1 \textmu g/ml) or high (10 \textmu g/ml) FSH concentrations. Overall, \textit{FSHR} mRNA levels were lower in whole ovaries during the NB compared with breeding season but were similar in immature COCs, whereas \textit{ESR2} levels did not differ in either group between intervals. We observed changes in \textit{PTGS2}, \textit{EGR1}, and \textit{EGFR} mRNA expression patterns across maturation in COCs within but not between the two seasons. The lack of seasonal differentiation in FSH-related genes was not consistent with the decreased developmental capacity of oocytes fertilized during the NB season. These findings reveal that the seasonal decrease in cat oocyte sensitivity to FSH occurs both \textit{in vivo} and \textit{in vitro}. Furthermore, this decline is unrelated to changes in expression of \textit{FSHR} mRNA or mRNA of FSH-induced genes in COCs from antral follicles.

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Introduction

The female domestic cat clearly and consistently displays seasonal reproductive activity with increasing northern latitudes (Jemmett & Evans 1977, Hurni 1981, Spindler & Wildt 1999, Freistedt \textit{et al} 2001, Comizzoli \textit{et al} 2003, Wallace & Levy 2006). In general, the nonbreeding (NB) season for free-living North American cat populations occurs over a 4 mo interval (Jemmett & Evans 1977), usually involving the months from August through January (Jemmett & Evans 1977, Spindler & Wildt 1999, Comizzoli \textit{et al} 2003, Wallace & Levy 2006). Previous studies have revealed that circulating gonadotropins, estrual activity, and pregnancy success decline in response to decreased photoperiod (Leyva \textit{et al} 1989, Joche & Joche 1993, Wallace & Levy 2006). Additionally, intraovarian cat oocytes are less responsive to FSH \textit{in vitro} during this quiescence period (Comizzoli \textit{et al} 2003). Our laboratory has demonstrated that cumulus–oocyte complexes (COCs) recovered during seasonal anestrus require ten times more supplemental FSH to achieve \textit{in vitro} maturation (IVM) and developmental competence compared with those collected during the breeding season (Comizzoli \textit{et al} 2003). Specifically, higher FSH concentrations added to IVM culture during seasonal anestrus increase the percentages of oocytes a) reaching metaphase II, b) fertilized, and c) fertilized oocytes reaching the blastocyst stage of development (Comizzoli \textit{et al} 2003). The requirement of COCs for increasingly more FSH during the period of reproductive quiescence suggests that there is a seasonally mediated differentiation (or aberration) in mechanism(s) within the ovarian follicle that perceives and accommodates the FSH signal.

FSH acts through the FSH receptor (FSHR) that is primarily expressed by cumulus and mural granulosa cells (sheep, Tisdall \textit{et al} 1995; pig, Liu \textit{et al} 1998; cat, Saint-Dizier \textit{et al} 2007). Seasonally mediated variation in FSHR expression (mRNA or protein) has only been demonstrated in males of a few species (black bear, Howell-Skalla \textit{et al} 2000; bat, Hayashi \textit{et al} 2002;
sheep, Sanford et al. 2002) where reductions are directly correlated with spermatogenic declines. Therefore, this relationship is interesting to examine, especially given knowledge that a decrease in FSHR density has the potential of altering the response of rat granulosa cells to FSH from being stimulatory to antagonistic (Donadeu & Ascoli 2005). Additionally, human IVF patients that present as ‘poor responders’ to FSH treatment have been shown to have lower ovarian FSHR levels (Cai et al. 2007).

It is known that FSHR undergoes multiple splicing in most species that have been studied (human, Kelton et al. 1992; sheep, Yarney et al. 1993; rat, Kraaij et al. 1998; mouse, Tena-Sempere et al. 1999; bovine, Calder et al. 2003), including in the cat (Genbank AY524543.1, AY521181.1). Two isoforms of FSHR mRNA exist in the latter species, a full-length (FL-FSHR) and a truncated (Tr-FSHR) transcript missing exon 3 (Genbank AY524543.1, AY521181.1) that contains the signal peptides for a site involved in FSH binding (Heckert et al. 1992, Fan & Hendrickson 2005). Although the physiological role of FSHR splice variants remains unclear for most species, truncated or mutated transcripts have been shown to function as dominant negative receptors in the sheep (Sairam et al. 1996) or to interfere with the translation andimerization of wild-type FSHRs in the human (Gerasimova et al. 2010, Lalioti et al. 2010).

Seasonally mediated FSH efficacy may also result from altered FSHR function as well as changes in expression level. An earlier study has identified potential markers of developmental competence in cumulus cells that are downstream of FSH action (Assidi et al. 2008). This includes genes regulating cumulus expansion and ovulation (McKenzie et al. 2004, Tamba et al. 2008), as such prostaglandin-endoperoxide synthase 2 (PTGS2 or COX2), that encodes the enzyme controlling the rate-limiting step in prostaglandin synthesis (Takahashi et al. 2006) essential for coordinated oocyte maturation and ovulation (Lim et al. 1997, Matsumoto et al. 2001, Gaytan et al. 2006, Duffy et al. 2010). PTGS2 is positively regulated by FSH, epidermal growth factor (EGF; Joyce et al. 2001, Shimada et al. 2006), and oocyte-secreted factors (Elvin et al. 2000, Joyce et al. 2001, Dragovic et al. 2007). Because inhibiting PTGS2 during IV can decrease cumulus expansion, oocyte maturation, and ultimately embryo production (Nuttinck et al. 2011), we predicted that a seasonal difference in the production of resulting enzyme may indicate altered FSH function. EGF can promote oocyte maturation (Farin et al. 2007, Hatoya et al. 2009, Uhm et al. 2010) and is necessary for FSH-induced meiotic resumption (Chen et al. 2008). This is most likely achieved through FSH-induced upregulation of EGF receptor (EGFR) mRNA expression in cumulus cells (bovine, Assidi et al. 2008; goat, Almeida et al. 2011). Given this direct relationship between FSH and EGFR expression, this gene was chosen as a direct indicator of FSH efficacy. A gene associated with the latter stages of oocyte maturation, early growth response protein-1 (EGR1), has been suggested to directly correlate with improved embryo outcome (Robert et al. 2001). EGR1 mRNA is closely linked to emergence of LH receptors (LHR; Yoshino et al. 2002), is induced by FSH and LH (Russell et al. 2003), and increases in response to human chorionic gonadotropin (hCG) both in vitro (Yoshino et al. 2002) and in vivo (Espey et al. 2000). As LHR expression is subsequent to FSH action, EGR1 might be considered as a late-stage indicator of FSHR function. We also predicted that altered levels of PTGS2, EGFR, and EGR1 mRNA potentially observed seasonally in cat cumulus cells could be ameliorated by increasing FSH concentrations during IVM. While we speculated that altered FSHR function was the likely cause of seasonal fluctuations in oocyte FSH sensitivity, we also predicted that it would be an oversight to exclude a pathway known to synergize with FSH. Therefore, we also measured estrogen receptor β (ESR2), a receptor coactivated by FSH in granulosa cells (Kouzu-Fujita et al. 2009) and which likely plays a role in directly regulating estrogen actions (Pasapera et al. 2005), especially meiotic resumption (bovine, Beker et al. 2002, Beker-van Woudenberg et al. 2004; canine, Kim et al. 2005; cervidae, Siriaroonrat et al. 2010; porcine, Kim et al. 2011).

This study takes advantage of the seasonal domestic cat model to examine the significance of differential ovarian responsiveness and gonadal success in the context of FSHR isoforms and function. Our objective was to better understand the seasonal and FSH-induced changes in gene expression in cat COCs, specifically by examining levels of FSHR mRNAs and genes known to be upregulated in other species by FSH (PTGS2, EGFR, and EGR1) or that synergize with FSH (ESR2) during oocyte maturation. For the NB season, we proposed that a) FSHR mRNA would decrease, possibly with increased Tr-FSHR compared with FL-FSHR; b) expression of PTGS2, EGFR, and EGR1 mRNA post-maturation would decrease while ESR2 mRNA remained stable; and c) that seasonal deviations in FSH sensitivity during maturation in vitro occurred by similar mechanisms in vivo.

**Results**

**Influence of simulated and natural seasons on number and quality of COCs before and after maturation**

After exogenous gonadotropin stimulation of cats maintained in long light (14 h, simulated breeding season) vs short light (8 h, simulated NB season) cycles, we aspirated similar (P>0.05) numbers of COCs per cat and proportions of expanded COCs (Table 1). The oocytes from these two distinctive groups also had the same (P>0.05) ability to achieve nuclear maturation (~60%). However, 84% of oocytes recovered from cats...
living under a long-day cycle fertilized with >60% advancing to morulae compared with only 48.5% and <10%, respectively, for oocytes from short light cycle counterparts (P<0.05; Table 1). No cell division was observed in oocytes used as parthenogenic activation controls (n=11 oocytes total, B and NB).

When the ovaries of random cats from spay clinics were examined during the two natural seasons (exposure to environmental photoperiod), there was no difference (P>0.05) in the proportion of total immature oocytes recovered at the germinal vesicle (GV) or GV breakdown (GVBD) stage (Table 2). Furthermore, in the presence of either the low or high supplemental FSH dose nearly, 80% of all cultured oocytes achieved nuclear maturation (MII) (P>0.05; Table 2). There was no effect (P>0.05) of FSH concentration on the kinetics or incidence of nuclear maturation (P>0.05) in these oocytes from cats experiencing natural light exposures during the two different times of the year. However, oocytes recovered during the breeding season had similar (P>0.05) developmental competence (fertilization and morulae formation) regardless of the FSH concentration used during maturation (Table 2). Furthermore, the poor ability of these oocytes from the NB period to fertilize and develop was improved (P<0.05) by supplementing with higher FSH (Table 2). No cell division occurred in parthenogenic controls (n=15).

Gene expression in whole ovaries and COCs in the breeding vs NB seasons

We identified two FSHR mRNA splice variants in the cat ovary, and both were expressed in the breeding and NB seasons (Fig. 1A, B and C). Sequence analysis confirmed that the shorter transcript was missing exon 3. In the whole ovarian analysis, there was a consistent trend for lower gene expression during the NB vs breeding season, but the only significance (P<0.05) occurred with overall FSHR mRNA expression; there was no statistical variation (P>0.05) in Tr-FSH, FL-FSHR, or ESR2 mRNA expression in immature COCs of cats exposed to natural light conditions (Fig. 1C). Furthermore, there was no such variation between seasons for the reportedly FSH-induced genes (PTGS2, EGR1, and EGFR mRNA) in COCs aspirated from preovulatory follicles of cats under simulated light conditions or immature oocytes of cats under natural photoperiod that were matured in vitro with 1 μg/ml FSH (Fig. 2A and B). Incubation of immature COCs from the NB period in the higher 10 μg/ml FSH resulted in variable (P>0.05) PTGS2, EGR1, and EGFR mRNA expression, and overall mean levels were similar to those measured when using the 1 μg/ml FSH treatment (Fig. 2C).

**Table 1** Seasonal differences in developmental capacity of oocytes collected after ovarian stimulation in cats maintained in long light (14 h, breeding) vs short light (8 h, nonbreeding) cycles.

<table>
<thead>
<tr>
<th>Season</th>
<th>Average no. COCs/cat (mean ± s.d.)</th>
<th>Expanded COCs/total COCs (% per cat)</th>
<th>MII oocytes/total oocytes (%)</th>
<th>Fertilized oocytes/total MII oocytes (%)</th>
<th>Morula/total fertilized oocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breeding (14 h)</td>
<td>29.3 ± 15.0</td>
<td>80.7</td>
<td>65.3</td>
<td>84.0ab</td>
<td>61.9a</td>
</tr>
<tr>
<td>Nonbreeding (8 h)</td>
<td>23.8 ± 7.6</td>
<td>65.7</td>
<td>57.1</td>
<td>48.5b</td>
<td>6.3b</td>
</tr>
</tbody>
</table>

Letters indicate significant differences between seasons within columns at P<0.05; COC, cumulus–oocyte complex; MII, metaphase II.

**Table 2** Nuclear maturation and developmental competence of oocytes collected from random cats subjected to ovariohysterectomy throughout the breeding vs nonbreeding seasons.

<table>
<thead>
<tr>
<th>Season</th>
<th>n</th>
<th>GV (%)</th>
<th>GVBD (%)</th>
<th>MI (%)</th>
<th>MII (%)</th>
<th>n</th>
<th>Fertilized oocytes/total MII oocytes</th>
<th>Morulae/fertilized oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breeding</td>
<td>36</td>
<td>75.0a</td>
<td>16.7a</td>
<td>2.8a</td>
<td>5.6a</td>
<td>32</td>
<td>66.7a</td>
<td>70.0a</td>
</tr>
<tr>
<td>Pre-IVM FSH 1 μg/ml</td>
<td>26</td>
<td>0.0b</td>
<td>0.0b,c</td>
<td>11.5c</td>
<td>88.5b</td>
<td>29</td>
<td>71.4a</td>
<td>65.0a</td>
</tr>
<tr>
<td>FSH 10 μg/ml</td>
<td>33</td>
<td>0.0b</td>
<td>0.0b,c</td>
<td>12.1a</td>
<td>87.9b</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonbreeding</td>
<td>Pre-IVM FSH 1 μg/ml</td>
<td>23</td>
<td>78.3a</td>
<td>17.4a</td>
<td>4.3a</td>
<td>0.0a</td>
<td>24</td>
<td>40.0b</td>
</tr>
<tr>
<td>FSH 10 μg/ml</td>
<td>29</td>
<td>3.4b</td>
<td>6.9b,d</td>
<td>10.3a</td>
<td>79.3b</td>
<td>31</td>
<td>64.3a</td>
<td>61.1a</td>
</tr>
</tbody>
</table>

a,b,c: Within each column, values with dissimilar superscripts are different at P<0.05; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II; IVM, in vitro maturation.
in vitro-matured COCs collected from spay material. Within the breeding season, COCs from preovulatory follicles expressed more \((P<0.05)\) PTGS2 mRNA than immature counterparts (Fig. 3A). However, levels did not increase in immature COCs after IVM with 1 or 10 \(\mu\)g/ml FSH (Fig. 3 B and C). PTGS2 mRNA expression was similar \((P>0.05)\) among mature COCs aspirated from follicles in vivo and in vitro-matured oocytes exposed to the different FSH doses (Fig. 4A, B and C). By contrast, EGR1 mRNA levels were lower \((P<0.05)\) in COCs recovered from preovulatory follicles in vivo and after IVM with 10 \(\mu\)g/ml FSH compared with immature COCs (Fig. 3A and C). There were no other differences \((P>0.05)\) in expression of this gene among other groups (Figs 3B and 4). Preovulatory COC levels of EGFR mRNA were similar \((P>0.05)\) to that measured in COCs that were immature (Fig. 3A) or matured with 1 \(\mu\)g/ml FSH (Fig. 4B) but were higher \((P<0.05)\) than oocytes exposed to 10 \(\mu\)g/ml FSH (Fig. 4C). Levels of EGFR mRNA were similar in COCs after culture with 1 and 10 \(\mu\)g/ml FSH and lower \((P<0.05)\) than pre-IVM levels (Figs 3B, C and 4A).

Within the NB season, PTGS2 expression was similar to that observed for the reproductively active interval. That is, COCs aspirated from preovulatory follicles expressed more \((P<0.05)\) PTGS2 mRNA than immature COCs but not after IVM with 1 or 10 \(\mu\)g/ml FSH (Fig. 3D, E and F). PTGS2 mRNA expression did not differ \((P>0.05)\) between IVM groups (Fig. 4A) or post-IVM compared with aspirated preovulatory COCs (Fig. 4E and F). PTGS2 expression was similar to that observed for the reproductively active interval.

**Figure 1** Seasonal variation in FSHRs and ESR2 mRNA expression in whole cat ovaries (B) and immature COCs (C). Sequence analysis confirmed two splice variants of FSHR mRNA in cat cumulus cells (A). 100 bp ladder; values are mean \(\pm\) S.E.M.; \(*P<0.05\); COC, cumulus-oocyte complex; FSHR, FSH receptor; ESR2, estrogen receptor 2; FL, full-length; Tr-, truncated; B, breeding season; NB, nonbreeding season; Rel., relative.

**Figure 2** Seasonal comparison of PTGS2, EGR1, and EGFR mRNA expression in preovulatory COCs (A) and after in vitro culture with 1 \(\mu\)g/ml (B) or 10 \(\mu\)g/ml FSH (C). Values are mean \(\pm\) S.E.M. PTGS2, prostaglandin-endoperoxidase synthase 2; EGR1, early growth response 1; EGFR, epidermal growth factor receptor; B, breeding season; NB, nonbreeding season; Rel., relative.
mRNA expression was highly variable in COCs of preovulatory follicles and appeared even more so in COCs matured with 10 µg/ml FSH. EGR1 mRNA was lower (P<0.05) in preovulatory follicles and after IVM with 1 µg/ml FSH compared with immature COCs (Fig. 3D and E). We observed no difference in other group comparisons for this gene (Figs 3F and 4). EGFR mRNA expression did not differ in group comparisons (Figs 3D, E and 4D, E).

Discussion

This study determined that the seasonal difference observed in cat oocyte response to FSH and capacity to mature and fertilize (Comizzoli et al. 2003) was not due to altered expression of FSHRs. We were unable to find any significant time trends in expression of the two known splice variants of FSHR that related to oocyte maturation. Furthermore, we discovered that the deficit in FSH responsiveness in cat oocytes in vitro was not due to seasonal changes in cumulus cell expression of several genes linked to FSHR function, specifically, PTGS2, EGR1, EGFR, and ESR2. Equally important was determining that the seasonal compromises observed in cat oocytes matured in vitro also occurred in counterpart oocytes recovered from cats during the simulated NB season in vivo. That is, this phenomenon was physiological and not an artifact of the culture system.

Whole ovaries in our study displayed a decrease in overall FSHR mRNA expression during the NB season. This striking variation may have resulted from differences in the proportions of follicles at various stages within whole ovaries between seasons, as in the cat, FSH binding has been demonstrated to increase with follicle (Saint-Dizier et al. 2007). In parallel, the overall decrease in FSHR mRNA we observed during the NB season has not been observed in isolated immature COCs (Comizzoli et al. 2003). A differential expression of FSHR mRNA between mural and cumulus granulosa cells could be hypothesized, but no difference on FSH binding was demonstrated between those two sites in cats (Saint-Dizier et al. 2007).

Our study confirmed observations (Genebank AY524543.1, AY521181.1) that there were two FSHR splice variants in cat reproductive tissues, and we determined that these were expressed by isolated COCs. Contrary to our hypothesis and previous studies in seasonal male species (black bear, Howell-Skalla et al. 2000; bat, Hayashi et al. 2002; sheep, Sanford et al. 2002), expression of these isoforms did not differ between reproductively active and inactive stages in the female cat. Therefore, this observation suggested that Tr-FSHR did not have a regulatory function in seasonal FSH sensitivity in the latter species. But, mRNA abundance does not always correlate directly with protein abundance or half-life (Maier et al. 2009), a situation that
could exist for both FL-FSHR and Tr-FSHR mRNAs. Although direct correlation of mRNA with protein content has not been demonstrated for FSHR isoforms, truncated splice variants are translated into protein in the human (Gerasimova et al. 2010), mouse (Tena-Sempere et al. 1999), and sheep (Yarney et al. 1997). In the case of women with poor FSH responsiveness, three abnormal FSHR transcripts have been identified that are coexpressed with wild-type FSHR (Gerasimova et al. 2010). These FSHR splice variants are unable to generate intracellular increases in cAMP, as is also the case for truncated transcripts in sheep (Rozell et al. 2009). It is hypothesized that these truncated mRNA variants may interfere with wild-type FSHR internalization, translation, or transcriptional regulation (Gerasimova et al. 2010).

As our mRNA data suggest that there was no seasonal variation in FSHR protein in the cat ovary, earlier observed differences in FSH responsiveness could be explained by altered pathways that involve FSH promotion of oocyte maturation. FSH combined with estradiol can increase proliferative activity of pig cumulus cells in vitro at a level greater than that measured by FSH alone (Kawashima et al. 2008). FSH also indirectly potentiates the transcripational activity of ESR2 (Pasapera et al. 2005). Nevertheless, we were unable to identify in the cat any seasonal link between the FSHR pathway and estrogen-sensitive gene expression; we observed no difference in ESR2 expression in this study between breeding and NB periods.

Therefore, to assess FSHR function in more detail, we examined expression of genes that are directly (PTGS2 and EGFR) or indirectly (EGR1) induced by FSH. Abolition of Ptgs2 in mice results in reproductive failure at multiple physiological levels, most notably lack of ovulation and perturbed fertilization (Lim et al. 1997). Blocking prostaglandins (i.e. PTGS2) decreases cumulus expansion and slows the rate of embryo development in the in vitro bovine system (Nuttinck et al. 2011). By contrast, higher PTGS2 mRNA levels occur in the cumulus cells of human oocytes expressing the highest developmental capacity (Gebhardt et al. 2011). In cat COCs, we indeed observed increased PTGS2 mRNA expression after oocyte maturation in situ, which was consistent with earlier observations in the pig (Kawashima et al. 2008) and mouse (Segi et al. 2003). Nonetheless, the level of induction of PTGS2 transcription (post-eCG/hCG in intact cats) did not vary between seasons despite being clear compromises in oocyte developmental potential during both simulated and natural reproductively quiescent (compared to active) periods. This lack of differentiation in PTGS2 expression extended to COCs recovered during natural anestrus and supplemented in vitro with a low (1 µg/ml) FSH concentration, a treatment known to fail to circumvent

![Figure 4](image-url)
inferior developmental capacity (Comizzoli et al. 2003). Therefore, unlike reported for the cow (Assidi et al. 2008) and human (McKenzie et al. 2004, Gebhardt et al. 2011), PTGS2 expression was not an accurate reflection of oocyte quality either in vivo or in vitro.

EGR1, and specifically higher EGR1 expression in cumulus cells, has been described as an effective marker of oocyte developmental potential in cattle (Robert et al. 2001, Tesfaye et al. 2009). The cat response was in stark contrast to the bovine system, with amounts of EGR1 being lower in mature compared with immature COCs and across all treatments during the natural and simulated reproductively active and inactive intervals. This striking difference with other species may result from the comparatively early acquisition of LHRs in cat granulosa cells (e.g. the early antral stage; Saint-Dizier et al. 2007). In fact, this protein has been shown to be closely linked to the emergence of LHRs in rat granulosa cells (Yoshino et al. 2002), increasing in response to hCG both in vitro (Yoshino et al. 2002) and in vivo (Espey et al. 2000). We suspect that this same event is occurring in the cat but earlier, thereby resulting in the lower EGR1 expression during final stages of maturation. Although we did not display the data, we also observed no differences in EGR1 levels in immature COCs between reproductive periods, suggesting that this protein is not seasonally regulated in early antral follicles.

A previous investigation by others has determined that EGF receptor mRNA expression is upregulated in cumulus cells several fold by FSH (Assidi et al. 2008). In our study, EGF mRNA levels were independent of FSH stimulation and comparatively stable in mature COCs compared with immature COCs during the NB interval regardless of maturational environment (i.e. in vivo or in vitro). Furthermore, FSH stimulation in vitro at a ‘standard’ or ‘high’ concentration significantly reduced EGF levels during the reproductively active season. One might conclude that FSH does not induce EGF expression in the cat and is not integral to oocyte maturation in this species. However, Assidi et al. (2008) assessed amounts of EGF for only 6 h of IVM culture unlike the 26–28 h monitored in our study. It may be that there is transient variation in EGF profiles across maturational phases in the cat that is influenced by reproductive interval. Sustained EGF activation also appears important for final maturation and cumulus expansion in rat oocytes (Reizel et al. 2010). For example, the latter investigation determined the need for a minimum of 4 h culture to stimulate EGF activation in isolated follicles, with less time adversely influencing LH-induced GVBD and cumulus expansion. In our study, there was also a trend for higher EGF levels in COCs exposed to 10 µg/ml FSH in the natural NB (compared with breeding) season; this treatment is also known to improve oocyte maturation in this species during periods of seasonal anestrus (Comizzoli et al. 2003). Collectively, these observations suggested that perhaps higher FSH increased EGR1 transcript stability. However, as LH (and not FSH) is known to increase EGR1 transcript stability in ovarian epithelial cells in the human (Choi et al. 2005), it is more likely that increased levels of this gonadotropin simply delayed EGR1 induction in the cat system.

In summary, our data did not reveal direct and consistent relationships between oocyte developmental capacity and season changes in FSHR mRNAs or FSH-induced genes in the domestic cat. The absence of significant trends, including gene expression in isolated COCs, may indicate that there are other functions of FSHR activation far more important in seasonally regulating oocyte competence. It was also significant that we clearly demonstrated decreased developmental competence of oocytes during periods of decreased light exposure following gonadotropin stimulation (using an FSH analog, eCG). That is, although the same number of total as well as high-quality COCs can be recovered from cats held under short light cycles, these oocytes have less capacity to fertilize and develop into morulae despite having comparable nuclear maturational abilities. This finding has practical implications for oocyte ‘rescue’ from rare domestic cat genotypes and threatened or endangered felid species (Johnston et al. 1991). As many other felids are also seasonally reproductively active, our findings here provide guidance on the best time of year to recommend oocyte recovery. More importantly, these fundamental mechanistic data are crucial for improving hormonal therapies and culture environments to overcome the inherent maturational limitations of intrafollicular oocytes collected during anestrus. Finally, the findings of species specificities illustrate the complexities in regulation of oocyte developmental competence and reinforce the challenges to be expected in developing consistently effective protocols.

Materials and Methods

All described animal procedures were approved by the Animal Care and Use Committee of the Smithsonian’s National Zoological Park. Unless otherwise designated, laboratory chemicals and drugs were purchased from Sigma Chemical Company.

Collection of preovulatory COCs

To determine whether the seasonal differences in oocyte maturation observed in vitro also occurred physiologically in vivo, we examined the indicators of follicle size, nuclear status, fertilization, and embryo development as well as gene expression in COCs matured in situ under light conditions simulating natural photoperiods.

Adult, female cats (n=8; Liberty Research, Inc., Waverly, NY, USA; Harlan Laboratories, Inc., Indianapolis, IN, USA; and University of California-Davis, Davis, CA, USA) aged 9 mo to 6 yr were housed under controlled lighting conditions in pairs...
at the Smithsonian Conservation Biology Institute (Front Royal, VA, USA), fed once daily (Nutro Max Cat; Nutro Products, Inc., City of Industry, CA, USA), and given free access to water. Long-day conditions (i.e. a natural breeding season) were imposed by providing an artificial fluorescent light cycle of 14 h light:10 h darkness over a total of 3 mo. The NB season was provoked by exposure to 8 h light:16 h darkness for a total of 3 mo. Of the eight cats used, five were assigned to the NB light cycle, two to the breeding light cycle, and one was used under both cycles (with 10 mo between treatments). After a minimum exposure of at least 42 days to a given light treatment, each female was given a standardized gonadotropin regimen (Donoghue et al. 1992, Pelican et al. 2006) involving an i.m. injection of 150 IU pregnant mares’ serum gonadotropin (Leinco Technologies, St Louis, MI, USA) followed 85 h later by an i.m. injection of 100 IU hCG. COCs from antral (>2 mm diameter) follicles were harvested 24 h post-hCG by laparoscopic aspiration according to our earlier descriptions (Pelican et al. 2010). Recovery medium consisted of HEPES-buffered modified Ham’s F10 (Irvine Scientific, Santa Ana, CA, USA) supplemented with 2 mmol/l L-glutamine, 1 mmol/l pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.37 mg/ml heparin (sodium salt Grade 1A), and 4 mg/ml BSA (A9418; Pelican et al. 2010, Crosier et al. 2011). COCs with an expanded cumulus cell mass were rinsed in nonheparinized collection medium. COCs (n=90) were stored in RNAlater (Ambion, Inc., Austin, TX, USA) for mRNA analysis (B, 11; NB, 11), stripped of cumulus cells, and fixed in 70% (v/v) ethanol for nuclear maturation assessment (B, 10; NB, 6) as described below or transferred to culture medium for insemination in vitro (B, 17; NB, 35).

**Collection of whole ovaries and immature COCs**

Immature COCs were collected throughout the year from the ovaries (n=70) of adult, mixed-breed, domestic cats after routine ovariohysterectomy conducted at a local, commercial veterinary clinic. We based our definition of seasonality on the observed frequency of large antral follicles on ovaries at gross examination and the proportion of high-grade COCs was collected. Higher rates were observed during periods of the year with longer daylight hours. We referred to the collection months of November through January as the NB season whereas June through August period as breeding season. Pairs of ovaries were dissected from the reproductive tracts, and those with active corpora hemorrhagica or corpora lutea were discarded. A subset of ovaries (n=10) was dissected into quarters and frozen immediately in liquid nitrogen for later molecular assessment of ovarian gene expression (see below) between the breeding (B; n=5 ovaries from five females; June–August) and NB (n=5 ovaries from five females; November–January) seasons. These ovaries were collected for examination of FSHR mRNA expression across all follicle types and the impact of mural granulosa cell expression between the reproductive and nonreproductive intervals. Within 6 h of ovarioectomy, the remaining tissues were transported in a cold container (at 4–8 °C) to the laboratory while immersed in PBS supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. Within 16 h of excision, ovaries were sliced in Hank’s-buffered Minimum Essential Medium (H-MEM; Gibco Laboratories) supplemented with 25 mmol/l HEPES (Gibco), 1 mmol/l pyruvate, 2 mmol/l l-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 4 mg/ml BSA (A9418) to recover immature oocytes (Spindler & Wildt 1999, Comizzoli et al. 2003).

COCs were pooled on each experimental day and graded according to the morphological descriptions of Wood & Wildt (1997). Only Grade 1 COCs (oocytes with homogenous, dark cytoplasm surrounded by >5 layers compacted cumulus) were chosen for analysis and randomly allocated to a treatment group. To establish pre-IVM levels of FSH-induced genes in immature COCs, a total of 92 COCs (B, 45 COCs; NB, 47 COCs) were placed in 50 µl aliquots of RNAlater (Ambion) and stored at −20 °C until molecular assessment. Some Grade 1 COCs (B, 36; NB, 23) were manually stripped of cumulus cells by pipetting repeatedly though a small bore Pasteur pipette to assess pre-IVM oocyte nuclear maturation status. Oocytes were dried on glass slides and fixed in 70% (v/v) ethanol for a minimum of 1 h and then stained using Hoechst nuclear dye as described previously for this species (Comizzoli et al. 2003). Stained oocytes were observed using epifluorescence (Olympus BX41; Olympus Corporation, Tokyo, Japan).

**In vitro oocyte maturation**

To evaluate the impact of seasonal differences in FSH responsiveness on FSH-induced gene expression, we cultured immature COCs with 1 or 10 µg/ml FSH using previously established methods from our laboratory (Comizzoli et al. 2003). In brief, this protocol relied on using Eagle’s-buffered MEM as the IVM medium supplemented with 1 mmol/l pyruvate, 1 mmol/l l-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 4 mg/ml BSA (A4161). We supplemented the IVM medium with 1 µg/ml LH (NIDDK-oLH-25; National Hormone and Pituitary Program (NHPP), Torrance, CA, USA), 1 µg/ml estradiol, and either 1 or 10 µg/ml FSH (NIDDK-ovine FSH-18; NHPP). After rinsing COCs by passing through three 50 µl aliquots of fresh IVM medium, five to ten immature COCs were placed in any given 50 µl IVF drops and transferred to 40 µl IVF drops and incubated (38.5 °C, humidified air with 5% CO2) for 26–28 h. A total of 275 COCs (B, 118; NB, 157) was subjected to IVM with 60% COCs placed in 50 µl RNAlater aliquots and stored at −20 °C until all replicates were completed. The remaining COCs (B, 59; NB, 53) were stripped of cumulus cells, fixed to glass slides, and nuclear maturation status evaluated (as described above). There were nine replicates for each FSH treatment (1 vs 10 µg/ml FSH) within both the breeding and the NB seasons.

**Insemination of oocytes and culture of embryos in vitro**

Aspirated preovulatory COCs and in vitro-matured COCs were inseminated using the same protocol. COCs were rinsed through two 50 µl IVF drops and transferred to 40 µl insemination drops that were equilibrated under oil in humidified, 5% (v/v) CO2. Insemination medium consisted of modified Ham’s F10 (Irvine Scientific) supplemented with 2 mmol/l l-glutamine, 1 mmol/l pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 5% (v/v) fetal bovine serum.
Table 3 Primer sets for relative quantification of mRNA in cat cumulus-oocyte complexes (COCs).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source or accession no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Tm (°C)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSHR (all)</td>
<td>NM_001048014.1</td>
<td>GCTCCACCTACATTGACCTACCTG</td>
<td>GTAACGGATGCCTGCTGCTGCTG</td>
<td>64</td>
<td>163</td>
</tr>
<tr>
<td>FL-FSHR</td>
<td>NM_001048014.1</td>
<td>CCCAAGAGGACAAAGGATGACCA</td>
<td>TCTATCATCTCCGACAGTCA</td>
<td>58</td>
<td>165</td>
</tr>
<tr>
<td>Tr-FSHR</td>
<td>AY524543.1</td>
<td>CCAAGAGGACAAAGGATGACCA</td>
<td>CCTTTTCAATCTATATTTCCTCCG</td>
<td>58</td>
<td>142</td>
</tr>
<tr>
<td>ESR2</td>
<td>AY237535</td>
<td>TCTCGGACCTCTCCTCCCTTAA</td>
<td>TAGTAAATGGCTGGCTGCACAA</td>
<td>64</td>
<td>170</td>
</tr>
<tr>
<td>PTGS2</td>
<td>NM_001110449</td>
<td>GAAATGGCTGCGGGTCTACAG</td>
<td>ATCTGTGATCTTGACAGTGG</td>
<td>58</td>
<td>193</td>
</tr>
<tr>
<td>EGR1</td>
<td>NM_000685156</td>
<td>TCCACAGGGCTTGGTGCCCA</td>
<td>AAGTCTAGTGCTGCAGTGCG</td>
<td>58</td>
<td>227</td>
</tr>
<tr>
<td>EGFR</td>
<td>Sequenced</td>
<td>CTCCGTGCTCCGCTGCTGACTAT</td>
<td>ATCTGTAAGTCAGTGCTGCTG</td>
<td>58</td>
<td>184</td>
</tr>
<tr>
<td>ACTB</td>
<td>AB051104.1</td>
<td>CCGATT'TTACGCGGAGATGCC</td>
<td>GTTGAAGGGTGCTCCTGGTGAGTG</td>
<td>64</td>
<td>181</td>
</tr>
<tr>
<td>RPM5</td>
<td>Penning et al. (2007)</td>
<td>GTTCCGAGAGGGCGGAGCTTGG</td>
<td>CTCAGGCTCACAGGCC</td>
<td>58</td>
<td>61</td>
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<tr>
<td>YWHAZ</td>
<td>NM_003406.2</td>
<td>CTCCACAGGAGACGCGAA</td>
<td>TGTCATCACCACCCGCA</td>
<td>58</td>
<td>88</td>
</tr>
</tbody>
</table>

FL-, full length; Tr-, truncated; Tm, melting temperature.

(Invitrogen). Two to six mature COCs were placed in each insemination drop. Oocytes were inseminated with conspecific spermatozoa collected, evaluated, and cryopreserved from two colony donors as described previously (Howard et al. 1993, Pukazhenthi et al. 1999). A 10 μl aliquot of 2 × 10^6 thawed and motile spermatozoa per milliliter was added to each 40 μl insemination drop (Spindler & Wildt 1999, Comizzoli et al. 2003, 2004, Spindler et al. 2006, Pelican et al. 2010). To control for parthenogenetic activation, some drops were sham-inseminated using 10 μl of sperm-free insemination medium (Comizzoli et al. 2004). Five replicate inseminations were performed for each FSH treatment (1 vs 10 μg/ml FSH) within both the natural breeding and the NB seasons. Sixteen to 18 h post-insemination, presumptive embryos were manipulated with micropipettes to strip free cumulus cells and spermatozoa. Embryos were rinsed though two drops of fresh insemination medium and then cultured in the same medium at 38.5 °C in humidified air (5% v/v CO2) and examined daily. On day 7 (day 1 = 24 h post-insemination), each embryo was examined for developmental stage morphologically (Swanson et al. 1994, Comizzoli et al. 2006) and then mounted and dried to a glass slide before fixation in 70% (v/v) ethanol. Fixed embryos were stained with Hoechst 33342 before epifluorescence evaluation to estimate blastomere number and define embryo developmental stage, as described previously (Comizzoli et al. 2001). An oocyte was considered fertilized if there was evidence of two pronuclei within an oocyte or continued embryo growth.

**Quantitative real-time PCR**

Total RNA was extracted from ovarian pieces (quarter ovaries) using Trizol reagent (Invitrogen Corp.) as per manufacturer’s recommendations with the addition of 20 μg glycogen (Invitrogen) as a carrier. Before total RNA extraction, COCs from each technical replicate in vivo (i.e. per cat) were pooled. For immature and in vitro-matured COCs, three replicates were pooled across season (from the nine replicates noted earlier) to create three experimental replicates per season for qPCR analysis. Replicates were pooled to increase total mRNA extraction and decrease the likelihood of intra-seasonal effects. The Picopure RNA isolation kit (Applied Biosystems, Foster City, CA, USA) was used to extract total RNA from immature, in vitro-matured, and preovulatory COCs as per manufacturer’s instructions. cDNA was generated from 100 ng COC total RNA and 500 ng whole ovarian total RNA using 200 U Superscript III reverse transcriptase (Invitrogen) and 0.09 OD260 units of random hexamer (Invitrogen) incubated at 50 °C for 60 min. Gene-specific primers were designed using Netprimer (Premier Biosoft International, Palo Alto, CA, USA) to known domestic cat sequence (FSHRs, PTGS2, and EGR1), domestic dog sequence (ESR2), or sequenced domestic cat cDNA (EGFR; Table 3).

Stratagene’s Brilliant SYBR Green QPCR Core Reagent Kit (Agilent Technologies, Cedar Creek, TX, USA) was used for relative quantification of gene expression. Cycling conditions for qPCR involved a 10 min incubation at 95 °C followed by 95 °C for 30 s, Tm (Table 3) for 60 s and 72 °C for 30 s, repeated 40 times. Amplification was measured using an ABI 7900 (Applied Biosystems) unit. The efficiency of qPCRs ranged from 90 to 110% with the exception of Tr-FSHR, which could not be optimized beyond 86% efficiency. Specificity of primer pairs was confirmed by melting curve analysis and gel electrophoresis of qPCR products. Three technical replicates were performed for each gene assay and expression levels normalized to three reference genes (ACTB, RPM5 (Penning et al. 2007), and YWHAZ) and a passive reference dye (6-carboxyl-X-rhodamine; ROX).

**Statistical analysis**

Number of morulae was divided by number of inseminated oocytes to give an index of relative embryo developmental success. For comparison of nuclear maturation, fertilization, and embryo development success, percentages were calculated from pooled data from all replicates. Percentage data were compared using χ² testing of significance (Systat v12.00.08; Systat Software, Inc., Chicago, IL, USA) and considered significant at P<0.05. Average number of COCs per cat was compared using a Student’s t-Test (Systat v12.00.08; Systat Software, Inc., Chicago, IL, USA).

The Relative Expression Software Tool (REST v. 2009) was used to compare relative expression levels of genes of interest normalized to the three reference genes. Reaction efficiency of the qPCR for each reference gene and gene of interest was entered into the REST calculation; bootstrapping was set at 100 levels. Values were considered significant at P<0.05.

**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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