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Response of hen pre-recruitment ovarian follicles to follicle stimulating hormone, *in vivo*

Kahina Ghanem[a,c], A.L. Johnson[a,b,c,*]

[a] Interdisciplinary Program in Physiology, The Pennsylvania State University, University Park, PA 16802, USA
[b] Center for Reproductive Biology and Health, The Pennsylvania State University, University Park, PA 16802, USA
[c] Department of Animal Science, The Pennsylvania State University, University Park, PA 16802, USA

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ABSTRACT

In laying hens, pre-recruitment ovarian follicles (1–8 mm diameter) are arranged as a continuum of size and predicted maturity. Cyclic recruitment of a pre-recruitment follicle to the preovulatory stage begins, in part, by the ability of the granulosa cell (GC) layer to initiate responsiveness to follicle stimulating hormone- (FSH-) induced cyclic adenosine monophosphate. The objective of this study was to determine if increased circulating concentrations of FSH during the ovulatory cycle increase the number of recruited follicles, in a dose-dependent manner. Equine chorionic gonadotropin (eCG) was initially tested due to its FSH-like properties and long half-life. Laying hens were injected, i.m., with 0 or 100 IU eCG, and ovaries were collected 29 h later. Recruited follicles were initially identified based on incorporation of yellow yolk and a weight of 250–900 mg. Recruitment was subsequently confirmed by both incubating the GC layer for 3 h with recombinant human (rh) FSH to establish FSH-responsiveness and quantifying *P450 side-chain cleavage enzyme* (*CYP11A1*) *mRNA*. Additional hens were injected with 0, 30, 75, and 300 IU eCG to establish a dose–response. Because eCG exhibits some luteinizing hormone activity, FSH-induced recruitment was evaluated by injecting 0.1, 0.33, 0.66, 1 or 3.3 μg rhFSH. Ovaries were collected 29 h post-injection, and expression of *CYP11A1* mRNA was quantitated in GCs from recruited and pre-recruitment follicles. One hundred IU eCG induced recruitment of 2–8 follicles compared to a single follicle in control hens. In contrast to pre-recruitment follicles, incubated GC from eCG-recruited follicles had initiated differentiation, indicated by increased *CYP11A1* and rhFSH-induced *STAR* mRNA and progesterone. Equine CG and rhFSH each increased the number of recruited follicles in a dose-dependent manner. Further, *CYP11A1* mRNA was significantly increased in GC layers from recruited, compared to non-recruited, follicles. We conclude that FSH-responsiveness within the GC layer of each pre-recruitment follicle increases with follicle size, and propose that this establishes the order of daily follicle recruitment.

1. Introduction

The laying hen (*Gallus gallus domesticus*) represents a particularly good model to study avian ovarian follicle selection (cyclic recruitment) into the preovulatory hierarchy, as follicle recruitment occurs on a near-daily basis for periods as long as 1 year. Because the duration of each ovulatory cycle is 24 + h, the single, functional left ovary has evolved to contain follicles at all stages of development (Gilbert, 1971). Typically, the laying hen ovary contains 4–6 preovulatory follicles (Gilbert, 1971) and 30–100 pre-recruitment follicles all arranged into size and maturity hierarchy (Gilbert et al., 1983). Each day the largest preovulatory follicle is ovulated while the remainder of follicles advance within the hierarchy. The number of preovulatory follicles is kept essentially constant by the recruitment of the largest follicle from a cohort of pre-recruitment (6–8 mm diameter) follicles to become the smallest (9–12 mm diameter) follicle within the preovulatory hierarchy (Woods and Johnson, 2005). This process is referred to as cyclic recruitment.

A critical marker of cyclic recruitment is the initial responsiveness of the GC monolayer to follicle stimulating hormone (FSH) (Johnson, 2015). FSH-responsiveness initiates differentiation of the GC layer, which results in the initial capacity for steroidogenesis (Nitta et al., 1991; Tilly et al., 1991). Prior to recruitment, freshly collected GC from 1 to 8 mm follicles remain undifferentiated and fail to initiate cyclic adenosine monophosphate (cAMP) production in response to a 1 h challenge with recombinant human (rh) FSH compared to GC from the most recently recruited (9–12 mm) follicle (Johnson and Lee, 2016; Tilly et al., 1991), this despite evidence that FSH receptor (FSHR)

* Corresponding author at: Interdisciplinary Program in Physiology, The Pennsylvania State University, University Park, PA 16802, USA.
E-mail addresses: kug163@psu.edu (K. Ghanem), alj14@psu.edu (A.L. Johnson).

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mRNA and protein are expressed as early as the 1–2 mm stage of development (Woods and Johnson, 2005; You et al., 1996). Consequently, GCs from pre-recruitment follicles essentially lack cAMP-dependent steroidogenic acute regulatory protein mRNA (*STAR*) expression (Bauer et al., 2000) and cytochrome p450 side-chain cleavage enzyme (CYP11A1) activity (Nitta et al., 1991; Tilly et al., 1991). There is published evidence that the inability of GCs to generate cAMP in response to FSH, *in vitro*, is mediated through a tonic inhibitory effect of the mitogen-activated protein kinase (MAPK) and/or protein kinase C pathways (Johnson et al., 2002; Woods et al., 2007; Woods and Johnson, 2005). Nevertheless, it was demonstrated that undifferentiated GCs from pre-recruitment follicles as small as 1–2 mm will become sensitized to FSH and undergo initial differentiation, *in vitro*, within 3 h after removal from the follicle and become fully differentiated within 24 h of culture (Johnson and Lee, 2016; Kim et al., 2013; Ocon-Grove et al., 2012; Woods and Johnson, 2005). Such results suggest that cyclic recruitment may result from a rapid decline in inhibitory cell signaling rather than the comparatively slower process of gene transcription and translation. This led to a hypothesis tested herein that increasing levels of FSH, *in vivo*, will promote recruitment of more than one follicle. Accordingly, the objectives of this study were to establish: (1) if eCG and/or rhFSH will induce recruitment of multiple follicles within 29 h after a single injection, *in vivo*, in a dose-dependent manner; and (2) if the GC layer collected from such recruited follicles is FSH-responsive and has initiated differentiation similar to GCs from a spontaneously recruited follicle.

2. Materials and methods

2.1. Animals

Animal care and experimental protocols described herein were conducted in accordance to the Pennsylvania State University Institutional Animal Care and Use Committee. Sixty to 70-week-old single comb white Leghorn hens (*Gallus gallus domesticus*) from the Hy-Line W-36 commercial strain, laying clutches of 5 eggs or more, were used in this study. Hens were individually caged in laying batteries, with free access to feed (commercial standard) and water, with lights on from 00:00 h to 15:00 h. Birds were killed by cervical dislocation.

2.2. Reagents

Lyophilized equine chorionic gonadotropin (eCG) was purchased in 1000 IU vials from Sigma-Aldrich (St. Louis, MO, USA) and stored at 4 °C. Initially, eCG was re-suspended with 0.2 μm filter sterilized 0.1 M phosphate buffered saline (PBS) at a concentration of 1 IU/μL. Stock solutions were prepared in aliquots of 30, 75, 100, or 300 IU and stored at −80 °C, then were further diluted with PBS to a volume of 0.5 mL immediately prior to intramuscular (i.m.) injection. Recombinant human FSH (rhFSH) was obtained from the National Hormone & Peptide Program (Torrance, CA, USA) and stored at −80 °C until re-suspended. Initially, rhFSH was diluted in PBS at a concentration of 0.1 μg/μl, and stored at −80 °C. Immediately prior to injection rhFSH was diluted with PBS into 0.1, 0.33, 0.66, 1, or 3.3 μg doses in a total volume of 0.5 mL.

2.3. Ovarian follicle count and weights

To confirm previous reports that chicken ovarian pre-recruitment follicles are arranged into a discrete size hierarchy (Gilbert et al., 1983; Perry et al., 1983), ovaries were collected from 5 hens 8 h after onset of light. Follicles > 1 mm diameter were dissected out, counted and weighed. Small white follicles, with a weight < 20 mg, and a diameter between 1 and 2 mm were classified into 1–2 mm group. Larger white follicles, weighing between 20 and 89 mg, with a 3–5 mm diameter were classified into the 3–5 mm diameter group. Small yellow follicles, weighing 90–240 mg, with a 6–8 mm diameter were classified to the 6–8 mm group.

2.4. eCG and rhFSH injections

To initially investigate the effect of increased levels of circulating FSH on recruitment, eCG was utilized because of its FSH-like properties and relatively long half-life, *in vivo* (Alvarez et al., 2016). Hens that laid the first or second egg of a clutch were injected with 100 IU eCG (Johnson and Leone, 1985; Johnson et al., 1985) or PBS vehicle into the gastrocnemius muscle 8 h after onset of photoperiod, and ovaries were collected 29 h after injection. All follicles 1 mm and larger were collected and weighed. Follicles were initially considered to be the most recently recruited if the diameter was 9–12 mm, the weight was between 250 and 900 mg and contained yellow yolk similar to a pre-ovulatory follicle.

To establish if the number of recruited follicles is related to dose injected, additional laying hens that laid their first or second egg of a clutch were injected with PBS, 30, 75, or 300 IU eCG (Johnson and Leone, 1985; Johnson et al., 1985) or 0.1, 0.33, 0.66, 1, 3.3 μg rhFSH (Arce et al., 2014) 8 h after onset of light, and ovaries were collected 29 h after injection (N = 6 per dose). All follicles 1 mm and larger were collected and weighed. GC layers were isolated and excess yolk was removed by washing with cold Dulbecco's Modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA), then GC were homogenized in 0.5 mL TRIzol then stored at −80 °C for RNA extraction and *CYP11A1* quantification.

2.5. Granulosa cell incubations

To determine if GCs from recruited follicles had initiated differentiation similar to spontaneously recruited follicles, individual GC layers were collected, together with GCs from the largest pre-recruitment follicle (6–8 mm diameter, 150–200 mg) and from a pool of 3 medium-sized pre-recruitment follicles (100–150 mg). GC layers were prepared for short-term incubation as previously described (Johnson and Lee, 2016). Briefly, the whole GC layer was excised from the follicle and transferred into a 15 mL conical tube. Excess yolk was removed by washing in cold DMEM, then centrifuged at 100 × *g* for 30 s (4 °C) twice and a third time for 5 min. Between washes GC layers were re-suspended by gentle pipetting to remove any remaining yolk. After the last wash the GC layers were gently dispersed into small pieces using a 1 mL pipette, added to 12 × 75 mm polypropylene tubes at a density of approximately 5×10^5 cells per mL in 1.5 mL DMEM containing 2.5% fetal calf serum, 1% non-essential amino acids (Thermo Fisher Scientific), plus 1% anti-microbial plus anti-mycotic solution (Thermo Fisher Scientific). Dispersed GCs were pipetted into three aliquots (1.0 mL per tube), one incubated for 3 h with 10 ng rhFSH/mL, a second aliquot incubated without rhFSH for 3 h, while a third aliquot was immediately centrifuged at 300 × *g* for 5 min to serve as a control. All cells were subsequently lysed with Trizol then stored at −80 °C for RNA extraction. Incubated cells were subsequently collected and processed similarly. Medium from the incubated tubes was collected and stored at −80 °C until assayed for progesterone by ELISA (Cayman Chemical, Ann Arbor, MI, USA) (Kim and Johnson, 2016).

2.6. RNA extraction and real time qPCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol and stored at −80 °C. Subsequently, RNA (0.5 μg) was DNase treated using the RQI DNase kit (Promega, Madison, WI, USA) and reverse transcribed using Superscript IV reverse transcription kit (Invitrogen) according to manufacturer's protocol. Non-reverse transcribed samples were generated for each sample as negative controls to confirm absence of genomic DNA contamination. Real time quantitative PCR was performed using the 7500

Table 1
Gallus gallus-specific primer sequences.

Gene	NCBI accession	Sequence
STAR	NM_204686.2	FWD 5′-TGCCTGAGCAGCAGGGATTTATCA REV 5′-TGGTTGATGATGGTCTTTGGCAGC
CYP11A1	NM_001001756.1	FWD 5′-ACTTCAAGGGACTGAGCTTTGGGT REV 5′-AGTTCTCCAGGATGTGCATGAGGA
RPL19	NM_001030929.1	FWD 5′-GTACCTGAAGGTCAAGGGTAAC REV 5′-AGAGCTTCTTGCGAGCTTT

Fast Real-Time PCRsystem (Applied Biosystems, Foster City, CA, USA) in 10 µL reactions containing PerfeCta SYBR Green FastMix with Low ROX (Quanta Biosciences, Inc., Beverly, MA, USA), and primers (Table 1) at a final concentration of 300 nM. Primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Primer specificity was established by sequencing the single, amplified product, and efficiencies were calculated using the standard curve method and were determined to be between 95% and 100%. Reactions were performed for 40 cycles under the following conditions: denaturing 95.0 °C for 3 s, annealing 56.0 °C for 30 s, extension 72.0 °C for 30 s. The melt curve stage was run using default settings. Ribosomal protein L 19 (*RPL19*) was used as a reference gene after determining that its expression was not affected by stage of follicle development or FSH treatment. The qPCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

2.7. rhFSH ELISA

eCG is heavily glycosylated (Sugino et al., 1987), and therefore has an extended half-life compared to rhFSH (Alvarez et al., 2016). To establish the time-frame for rhFSH to enter and remain in the circulation after i.m. injection, hens that laid the first, second, or third egg of the clutch were injected with 1 µg rhFSH i.m., 9 h after onset of light Four birds were randomly selected for blood sampling via the wing vein 0.5, 1.5, 3, 4.5, 6, and 7.5 h after injection. Blood samples were collected once from four non-injected hens (0 h samples) and once from each wing from 4 additional hens at each timepoint without sampling any hen at two sequential time points. Plasma rhFSH was assayed using a human FSH ELISA (RayBiotech, Norcross, GA) according the manufacturers protocol. The detection range of this ELISA assay was 8–2000 pg/mL. This ELISA cross-reacts to an unknown extent with chicken FSH, and detected comparatively lower levels at T0 (Table 2) compared to a previously published report of circulating levels during the ovulatory cycle (Krishnan et al., 1993). Nevertheless, overall highest levels of FSH occurred 1.5 h after i.m. injection.

2.8. Statistical analysis

Data are expressed as mean ± SEM. For mean comparisons unpaired *t*-tests were performed to assess if there was a significant difference between independent means of continuous data that had only two means. When a comparison was conducted between more than two means an analysis of variance was performed followed by Tukey mean comparison test (Kutner et al., 2004). Relationships between variables were assessed by performing simple linear regression. Data was checked for normality and constancy of variance of the error terms and were transformed to meet normality requirement where necessary (Sheather, 2009). Level of significance was set at $\alpha = 0.05$; $0.05 < \alpha < 0.1$ was considered a tendency towards significance. All statistical analyses were performed using the statistical analysis software, SAS (SAS Institute Inc., 2002–2012, Cary, NC, USA).

Table 2
Plasma rhFSH (pg/mL) following injection of 1 µg, i.m.

Time after injection (h)	N	Mean (pg/mL)	SE mean (pg)	One way ANOVA p = 0.0001
0	4	125.8	22.8	b
0.5	4	664	169	a
1.5	4	1039	130	a
3.0	4	853.3	77.8	a
4.5	4	743.3	91.4	a
6.0	4	880	110	a
7.5	4	580.8	44.3	ab

3. Results

3.1. Pre-recruitment follicles are arranged into a size continuum

Pre-recruitment follicles were found to be arranged into a size continuum from 1 to 8 mm diameter (Fig. 1). The 1–2 mm diameter category contained significantly ($p < 0.0001$) more follicles than the 3–5 mm and 6–8 mm diameter category with 29 ± 2.06, 10.6 ± 2.29, and 9.6 ± 1.69, respectively.

3.2. Multiple recruited follicles induced by eCG injection

Injection of 100 IU eCG, i.m., induced recruitment of 2–8 follicles with a mean of 4.3 ± 0.9, and weights ranging from 286 to 903 mg (mean, 481 ± 26 mg) (Fig. 2). This occurred without altering the size hierarchy of established preovulatory follicles at a time 29 h after the injection. Of note, eCG blocked ovulation of the F1 follicle in 4 out of the 6 injections, which resulted in the F1 follicle being significantly heavier in the 100 IU injected animals in which ovulation was blocked, compared to control (16.82 ± 0.14 g vs. 14.61 ± 0.51 g, $p = 0.008$). The number of 6–8 mm pre-recruitment follicles was not significantly different between the PBS- and eCG-injected groups (9.0 ± 1.5 follicles versus 7.0 ± 0.9 follicles, respectively, $p = 0.3$).

3.3. Evidence for GC differentiation in recruited follicles

GC layers collected from spontaneously- (Control) or 100 IU eCG-recruited follicles expressed the late response gene, *CYP11A1*, at higher levels than the non-recruited follicles in control and eCG-treated animals ($p = 0.0218$ vs. $p < 0.0001$, respectively), plus significantly higher levels of the immediate response gene, *STAR* ($p = 0.0034$ vs. 0.0008, respectively) and secreted progesterone ($p = 0.04$ vs. 0.01, respectively) after incubation with 10 ng FSH/mL for 3 h (Fig. 3).

3.4. eCG and rhFSH promote follicle recruitment in a dose-dependent manner

Results from the 30 IU, 75 IU, 100 IU and 300 IU eCG injections were fit to a simple linear regression model, in which the dose-related increase was natural log (Ln) transformed. The number of recruited follicles increased as the dose of eCG increased (1 ± 0.0, 1.5 ± 0.2, 1.4 ± 0.2, 4.3 ± 0.9, 9.8 ± 1.7 follicles, after 0, 30, 75, 100, and 300 IU eCG respectively) with a slope of 1.1 ± 0.3 ($p < 0.001$) for each additional natural log increase of eCG. There was a tendency for the number of 6–8 mm pre-recruitment follicles to decrease for each natural log increase in IU eCG injected (9.0 ± 1.5, 11.3 ± 1.6, 11.2 ± 1.5, 7.2 ± 0.9, 3.2 ± 0.4 follicles; respectively) with a slope of −0.6 ± 0.4 ($p = 0.1$) (Fig. 4, Left panel). Similar to the 100 IU dose of eCG (discussed above), the 30 IU, 75 IU, and 300 IU doses blocked ovulation of the F1 follicle in 6 out of 6, 5 out of 6, and 6 out of 6, of the animals injected, respectively, without disrupting the hierarchal arrangement. To assess whether the effects of eCG could be replicated by FSH, hens were injected, i.m., with 0, 0.1, 0.33, 0.66, 1, or 3.3 µg rhFSH (Fig. 4, Right panel). This resulted in a significant increase in number of recruited follicles (1 ± 0, 1.8 ± 0.3, 2.5 ± 1.0, 2.6 ± 0.9, 8.2 ± 1.4 and 11.8 ± 1.7 follicles, respectively) with a slope of

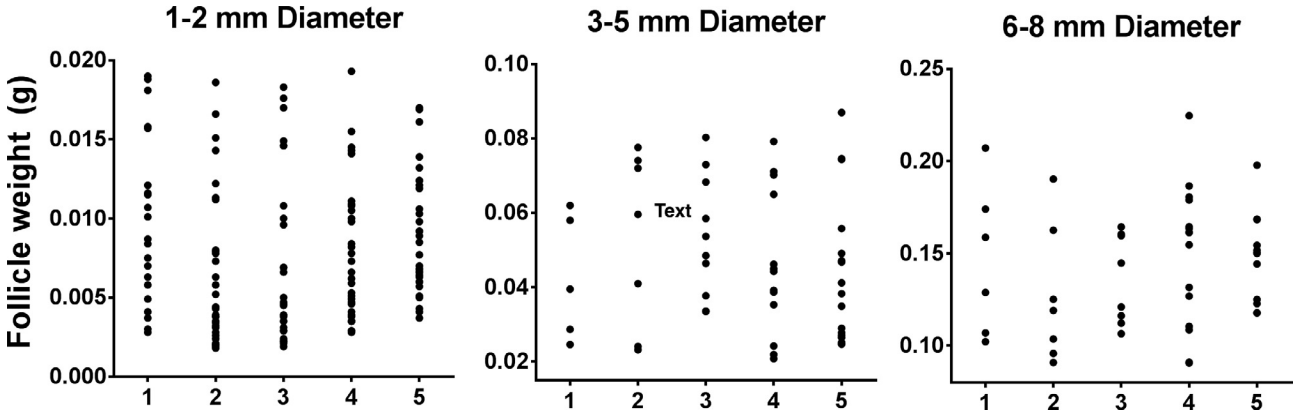

Fig. 1. Ovarian follicles throughout growth and development are arranged into a discrete size and weight hierarchy. Each number on the x-axis represents an individual hen, while each dot represents one follicle. Small white follicles, with a weight < 20 mg and a diameter between 1 and 2 mm, were classified as the 1–2 mm group. Follicles weighing between 20 and 89 mg with a 3–5 mm diameter were classified into the 3–5 mm diameter group. Follicles, weighing 90–240 mg, with a 6–8 mm diameter were classified as the 6–8 mm pre-recruitment group.

2.4 ± 0.4 follicles ($p < 0.0001$) for each additional natural log increase in μg of rhFSH injected. The number of 6–8 mm pre-recruitment follicles did not significantly decrease as the dose of rhFSH increased (7.3 ± 0.6, 8.3 ± 2.4, 10 ± 1.5, 9.9 ± 1.6, 8.2 ± 1.1, 4.3 ± 1.9 follicles, respectively; $p > 0.05$). Unlike eCG, rhFSH did not consistently block ovulation of the F1 follicle, except in two instances after injection of 0.33 μg rhFSH. Only in one hen was ovulation not observed after injection of the PBS control. In all instances, the differentiated status of GCs from the eCG- and FSH-recruited follicles was confirmed by the presence of significantly increased *CYP11A1* expression in freshly collected GC layers from recruited, compared to non-recruited, follicles (Fig. 5).

4. Discussion

We conclude that the GC layer from pre-recruitment follicles becomes more responsive to FSH with increasing size, and that increasing the dose of exogenous FSH initiates recruitment of multiple follicles into the preovulatory hierarchy in a dose-dependent manner. In an initial experiment, pre-recruitment follicles were confirmed to be

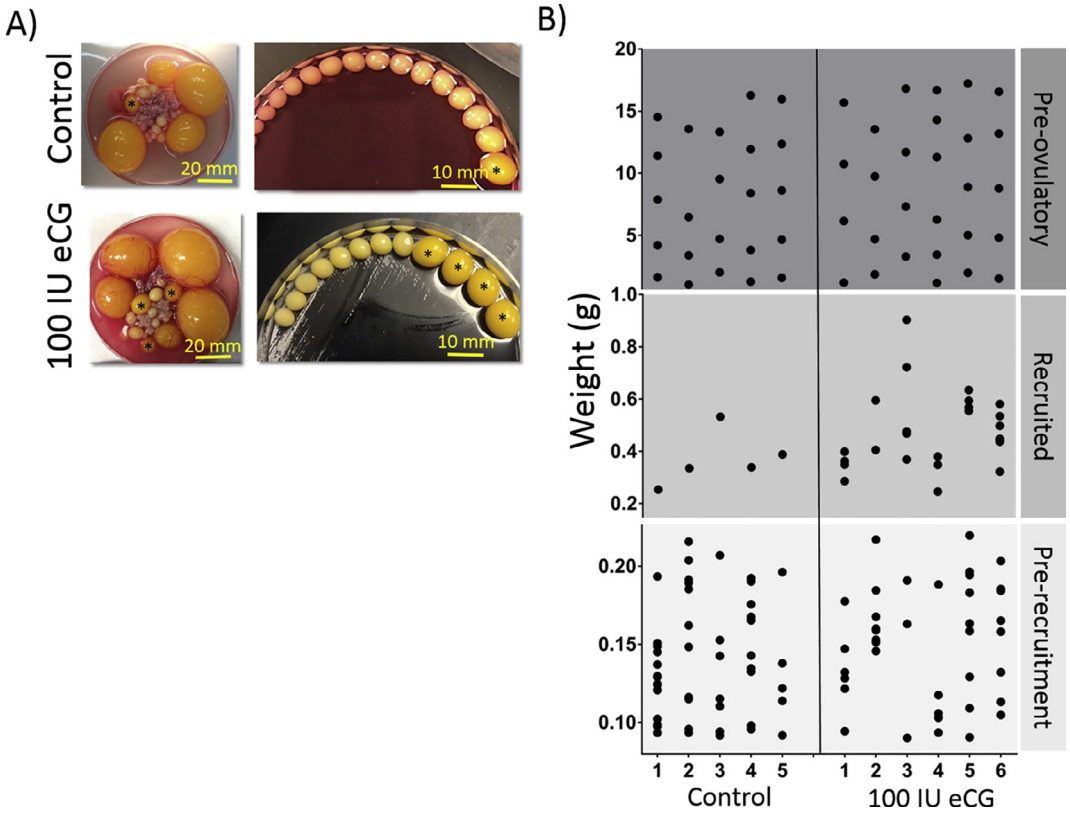

Fig. 2. (A) Top left. Ovary from a control-injected hen with four yolk-filled, preovulatory follicles with the arrangement of slow-growing follicles and the single, most recently recruited follicle (9–12 mm, *; Top right). Bottom left depicts an ovary containing four recently recruited follicles 29 h after injection of 100 IU eCG while the hierarchy of preovulatory follicles remains intact. Bottom right shows pre-recruitment follicles plus 4 recently recruited follicles (*). (B) Comparison of weight distribution and distribution of preovulatory, recruited, and pre-recruitment follicles between control (PBS)-injected (left) and 100 IU eCG-injected hens (right). N = 5 or 6 hens.

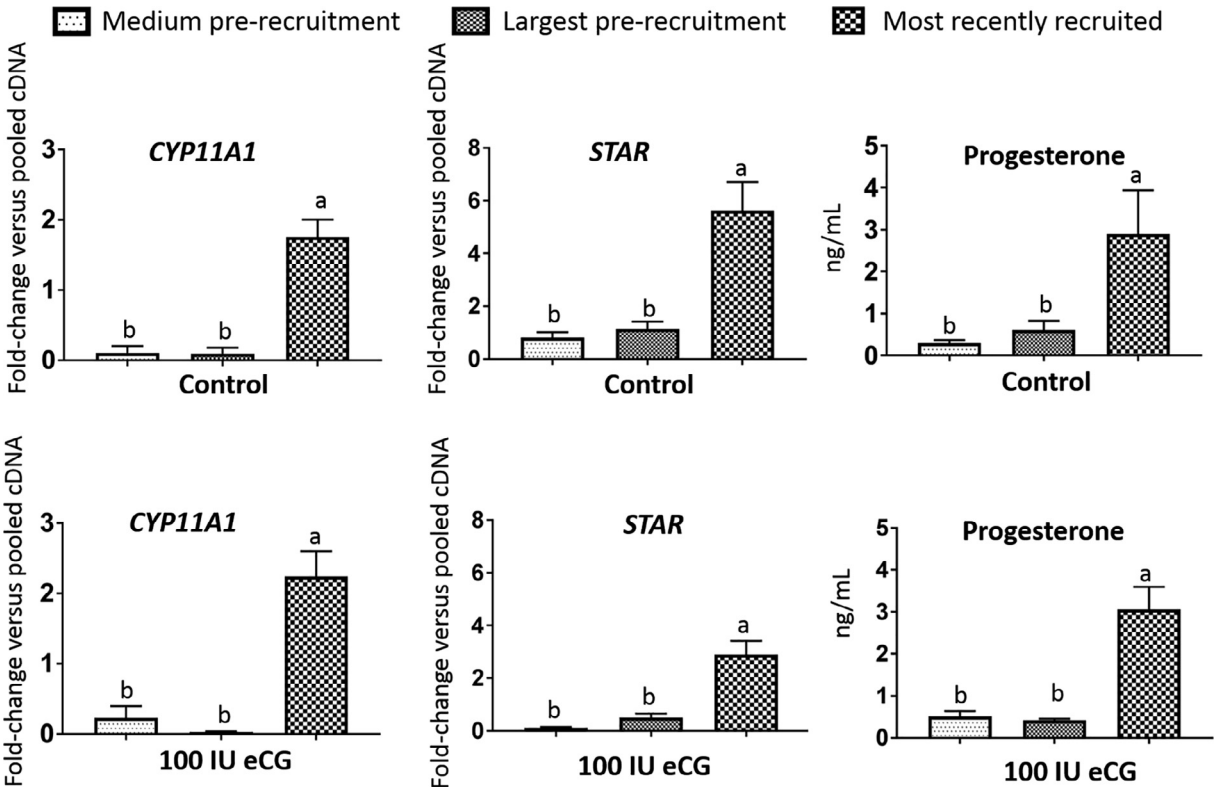

Fig. 3. Increased *CYP11A1* mRNA expression in fresh GC from recently recruited (9–12 mm) follicles compared to the largest and medium-size non-recruited follicles from both the control- (N = 3) and the 100 IU-eCG (N = 4) injected animals (a, b: p = 0.0218, < 0.0001, respectively). A 3 h challenge with 10 ng rhFSH, *in vitro*, increased *STAR* expression and in GC collected from 9 to 12 mm follicles in both control- (N = 3) and 100 IU eCG (N = 4) -injected animals compared to their respective largest and medium size pre-recruitment follicles (a, b: p = 0.0034, 0.0008). The 3 h rhFSH *in vitro* challenge also induced the GC of recently recruited follicles to secrete significantly higher concentrations of progesterone than GC from largest non-recruited and the medium-size non-recruited follicles from both the control- (N = 2) and the 100 IU-eCG (N = 3) injected animals (a, b: p = 0.04, 0.01, respectively).

arranged into a discrete size hierarchy (Fig. 1). Injection with a 100 IU of the FSH-like hormone, eCG induced recruitment of multiple follicles into the preovulatory hierarchy within 29 h (Fig. 2) and GCs from eCG-recruited follicles demonstrated steroidogenic capacity similar to that of a spontaneously recruited follicle (Fig. 3). To evaluate if the effect of eCG was dose-dependent and due to FSH bioactivity, additional hens were injected with 30, 75, or 300 IU eCG or 0.1, 0.33, 0.66, 1 or 3.3 μg rhFSH. Differentiation status of GCs from the recruited follicles was assessed via measurement of *CYP11A1* mRNA. Results demonstrate that increasing blood levels of FSH induces follicle recruitment in a dose-dependent manner and that FSH alone can account for this effect (Fig. 4). The differentiated status of GCs from recruited follicles was

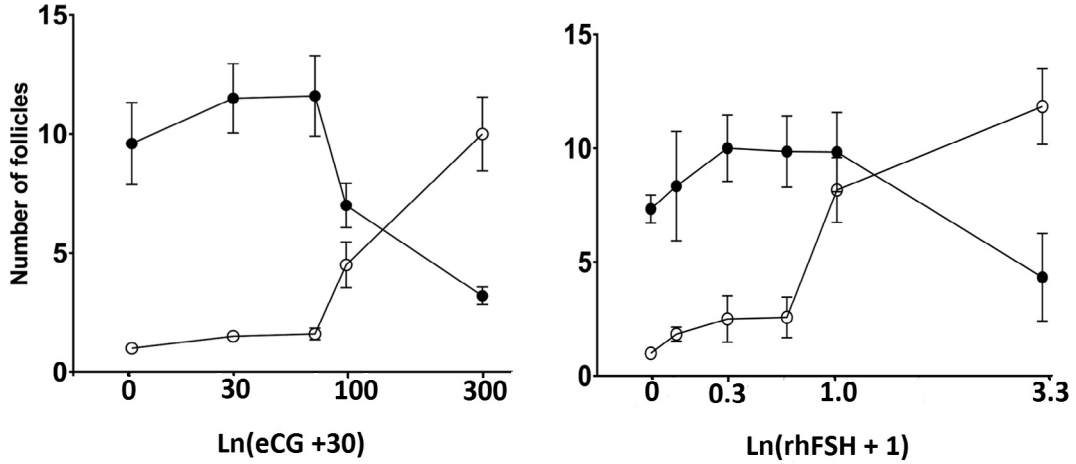

Fig. 4. Dose–response data were fit to a simple linear regression model, where the natural log of the dose + constant was calculated (constant was added to include 0 values in the analysis). Left. The number of eCG-recruited follicles (open circles) increased in a linear fashion following a slope of 1.1 ± 0.3 (p < 0.001) for each additional natural log increase of eCG. There was a tendency for the number of 6–8 mm pre-recruitment follicles (filled circles) to decrease for each natural log increase in IU eCG, following a slope of −0.6 ± 0.4 (p = 0.1) (N = 6/dose). Right. Increasing doses of rhFSH also resulted in a linear increase in follicle recruitment with following a slope of 2.4 ± 0.4 (p < 0.0001) follicles for each additional natural log increase in μg of rhFSH injected. The number of 6–8 mm pre-recruitment follicles did not significantly decrease as the dose of rhFSH increased (N = 6/dose).

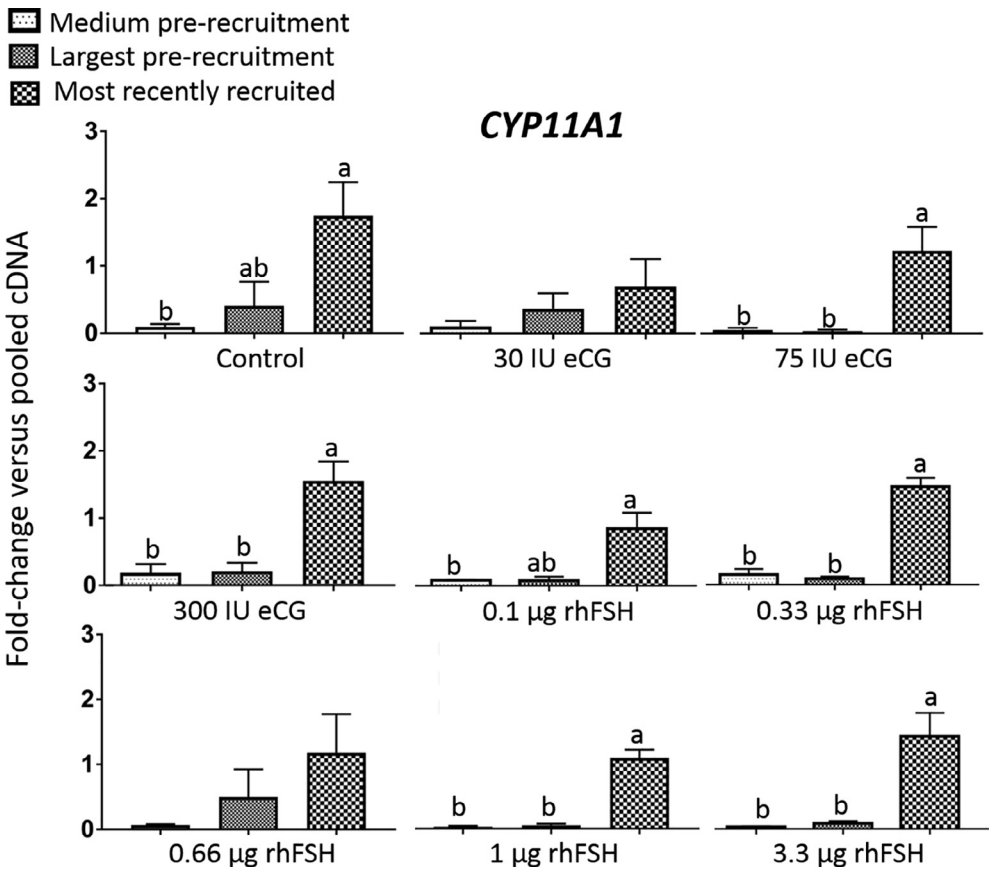

Fig. 5. *CYP11A1* mRNA expression in freshly harvested granulosa cells from recruited follicles, the largest pre-recruitment follicle, and the medium sized pre-recruitment follicles in control-injected animals or in response to 30 IU, 75 IU, and 300 IU eCG injection (one-way ANOVA; $p = 0.035$, 0.3472, 0.0037, 0.0094, respectively), or in response to 0.1, 0.33, 0.66, 1, and 3.3 µg rhFSH injection (one way ANOVA; $p = 0.031$, < 0.001, 0.252, < 0.001, < 0.001, respectively).

confirmed by increased expression of *CYP11A1* mRNA compared to their non-recruited counterparts (Fig. 5).

Although neither FSH nor eCG injection disrupted the hierarchal order of preovulatory follicles, each dose of eCG blocked ovulation of the F1 follicle with an incidence of 67–100%. A similar block of ovulation has been previously reported, and may be explained by eCG causing a decrease in the plasma luteinizing hormone (LH) concentration, thus, leading to insufficient levels of LH to induce ovulation (Johnson et al., 1984, 1985). Conversely, in the rhFSH treatment group, ovulation only failed to occur in 3 of 36 hens, twice after 0.33 µg rhFSH, and once in a control-injected animal. Thus, it is possible that in these 3 hens ovulation failed to occur for reasons unrelated to injection. The number of pre-recruitment follicles in eCG-injected animals tended to decrease as the number of recruited follicles increased ($p = 0.10$) (Fig. 4, Left panel, black solid circles), suggesting recruitment without replacement into the pre-recruitment (6–8 mm) follicle pool, yet rhFSH did not have the same effect. Instead, rhFSH appeared to stimulate growth of new pre-recruitment follicles as well as induce recruitment of multiple follicles into the preovulatory hierarchy. This is especially apparent following the 1 µg rhFSH injection, where both the number of recruited follicles and pre-recruitment follicles increased compared to control-injected hens. This stimulatory effect on the number of pre-recruitment follicles may have some threshold sensitivity, at which rhFSH no longer can stimulate the growth of replacement pre-recruitment follicles. This is apparent with the 3.3 µg injection (Fig. 4, Right panel). It is interesting to note that neither eCG nor rhFSH caused a disruption in the size hierarchy of pre-recruitment follicles.

In previous studies the stimulatory effects of FSH treatment within the hen ovary have been reported after multiple days of FSH injections (Mitchell, 1970; Nalbandov and Card, 1945; Opel and Nalbandov, 1961; Palmer and Bahr, 1992; Taber et al., 1958). To our knowledge, this is the first study to establish that a single injection of FSH is capable of initiating FSH-responsiveness within the GC layer, resulting in the dose-dependent recruitment of multiple follicles to the preovulatory hierarchy within a single ovulatory cycle. Moreover, this study establishes that each recruited follicle demonstrates morphological and cellular characteristics that exist within a follicle following a spontaneous recruitment, including increased size, yellow yolk incorporation and weight. This indicates that FSH alone is capable of mediating the process by which a pre-recruitment follicle is recruited into the preovulatory hierarchy. Importantly, it remains to be determined whether the timing of ovulation and follicle recruitment are always linked.

In summary, a fundamental difference exists between the hen and mammals regarding the status of FSH-responsiveness within the GC layer at the time of recruitment. Specifically, selection of the dominant follicle in monovulatory mammals is proposed to occur in the single follicle with the greatest FSH-responsiveness in the face of declining concentrations of circulating FSH (reviewed by Fortune et al., 2004). By comparison, results from *in vitro* studies in the laying hen (Johnson and Lee, 2015) and turkey hen (Ghanem and Johnson, 2018) together with *in vivo* studies presented herein indicate that follicle recruitment normally occurs in from a single pre-recruitment follicle following the initial capacity for FSH to stimulate signaling via cAMP within the GC layer. Investigations are ongoing to establish the most proximal cellular mechanism responsible for initiating FSH-responsiveness.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.10.004.

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