ABSTRACT

Title of Thesis: **OVARIAN STROMAL CELLS IMPROVE SURVIVAL, BUT NOT GROWTH, IN PRE- AND EARLY ANTRAL FELINE FOLLICLES**

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Ovarian stromal cells act as crucial support and regulators for *in vivo* folliculogenesis; however, less is known about their effect on *in vitro* grown follicles. The objective of this study was to investigate the impact of ovarian stromal cell co-culture or conditioned medium (CM) on survival and development of cat pre-, early, and antral follicles *in vitro*.

Ovaries were obtained from cats older than six months (n = 3), then enzymatically digested to release stromal cells. The ovarian stromal cells were allowed to grow to confluency in a T75 flask, before being cryopreserved for long term storage in liquid nitrogen. Cells were thawed one week prior to follicular culture onset, and passaged once before CM collection. CM was subsequently removed 24 - 48 hours after feeding, and stored at -80°C until used. Ovarian follicles were mechanically isolated from cats older than six months (n = 23 cats, 155 follicles), encapsulated in 0.5% alginate hydrogel. The isolated follicles were then divided into five treatment groups (control, ovarian stromal cell co-culture, 20% CM, 50% CM, and 100% CM in Endothelial Cell Growth Medium), and classified based on initial diameter as preantral (224.4 ±

4.7 μm), early antral (394.8 ± 7.4 μm), or antral (592.2 ± 18.8 μm). Culture subsequently lasted for 13 days, and survival and growth of the follicles were evaluated on Days 0, 4, 6, 8, 11 and 13. At the end of culture, follicles were assessed via qRT-PCR for expression of *CYP19A*, *FSHR*, and *GDP9* to further quantify development. Statistical analysis was done in R software.

Follicles in 100% CM had higher survival up to Day 11 of culture as compared to other treatment groups (Cox proportional hazards model, $p \leq 0.01$). Initial stage also influenced survival, with antral follicle survival significantly lower than that of pre- and early antral follicles ($p \leq 0.0001$). However, no differences in growth were detected across the treatment groups, nor across initial size classifications (Kruskal-Wallis test, $p > 0.05$). Post culture qRT-PCR analysis of the three selected genes showed upregulation of *CYP19A* in 50% CM follicles compared to the control (ANOVA, $p \leq 0.05$). However, there were no differences in *CYP19A* expression between the control and other treatment groups, or in *GDF9* and *FSHR* expression among culture groups ($p > 0.05$). In summary, the findings demonstrated that conditioned medium collected from primary culture of ovarian stromal cells improves *in vitro* survival and modulates *CYP19A* expression of isolated cat follicles. Further research to identify paracrine factors present in conditioned medium will elucidate the roles of ovarian stromal cells pertaining to follicle survival during *in vitro* folliculogenesis.

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by

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List of Abbreviations

2D – two-dimensional

3D – three-dimensional

Akt – protein kinase B

ALK – activin-like receptor kinase

AMH – anti-Mullerian hormone

ART – assisted reproductive technologies

BAX – BCL2-associated X protein

BMP – bone morphogen protein

BSA – bovine serum albumin

CASP3 – caspase 3

CD – cluster of differentiation

cDNA – complimentary DNA

CI – confidence interval

CL – corpus luteum

CM – conditioned medium

COC – cumulus oocyte complex

COX2 – cyclooxygenase 2

CX43 – connexin 43

D – degenerated

DAPI - 4′,6-diamidino-2-phenylindole

DNA – deoxyribose nucleic acid

EEP – European Endangered Species Program

EGF – epidermal growth factor

EGM – endothelial cell growth medium

EGM (-) – EGM without growth factors VEGF, IGF, and FGF

EPC – human endothelial progenitor cells

FBS – fetal bovine serum

FGF – fibroblast growth factor

Fox12 – Forkhead box L2

FSH – follicle stimulating hormone

FSHR – FSH receptor

GDF9 – growth differentiating factor 9

GH – growth hormone

GnRH – gonadotropin releasing hormone

GV – germinal vesicle

GVBD – germinal vesicle breakdown

HAS2 – hyalurononan synthase 2

hCG – human chorionic gonadotropin

HIV – human immunodeficiency virus

ID – initial diameter

IGF – insulin-like growth factor

IVF – *in vitro* fertilization

KIT - receptor tyrosine kinase

KITL – KIT ligand

LH – luteinizing hormone

LHR – LH receptor

LIF – leukemia inhibiting factor

MAPK – mitogen activated protein kinase

MEM – minimum essential medium

MII – metaphase II

mRNA – messenger RNA

mTOR – mammalian target of rapamycin

mTORC1 – mTOR complex 1

Nobox – newborn ovary homeobox protein

NT-4 – neurotropin 4

PBS – phosphate buffered saline

PDGFβ – platelet-derived growth factor beta

PGF2α - prostaglandin F2 alpha

PI3K – phosphatidylinositol 3-kinase

PKA – protein kinase A

qRT-PCR – quantitative real time polymerase chain reaction

RBC – red blood cell

RNA – ribonucleic acid

ROS – reactive oxygen species

s – second

SSP – Species Survival Program

StAR – steroidogenic acute regulatory protein

TGFβ – transforming growth factor beta

TNF – tumor necrosis factor

VEGF – vascular endothelial growth factor

Chapter 1: Literature Review

1.1. Introduction

With many species on the brink of extinction from global climate change, overhunting, or habitat loss, it is imperative to invest in research to preserve germplasm of genetically valuable individuals. One family of interest is *Felidae*. These carnivores play an important role in their habitats as keystone species, keeping the ecosystem in balance. If wild felids disappeared, prey species in their area would experience a population boom, crippling the land's ability to sustain them. In some areas, large felids such as lions, tigers, and leopards also create revenue for locals, as ecotourism brings in money and sustains jobs. Finally, by preserving all wild felids, we preserve the integral cultural impact they bring.

Ideally, all conservation efforts would be focused on *in situ* environments; however, this is not always possible. Historically, felid species have been resistant to extinction events. However, with continued human disturbance like poaching and habitat destruction, many populations are threatened (Swanson et al., 2007). Of the 39 species in the Felidae family listed on the IUCN Red List, 18 are listed as vulnerable or endangered, with the three biggest factors including habitat loss, hunting/trapping, and decline in prey species (*The IUCN Red List of Threatened Species*, n.d.). Therefore zoos, through the Species Survival Program (SSP) or the European Endangered Species Program (EEP), become important repositories for genetic banks via live animals and cryopreserved gametes.

These cryopreserved gametes are crucial, as zoos do not have the physical space and, more importantly, the funding necessary to maintain a genetically diverse population for every endangered species through housing live animals. In fact, maintaining 95% genetic diversity

from a group of founders over 200 years would require breeding nearly double the animals needed for a 90% genetic diversity (Conway, 1986). This population constraint for all endangered species is unsustainable for even a network of zoos. Should genetic diversity decrease in captive populations, they would be subjected to genetic drift, inbreeding, and lowered fitness for the species' ability to survive if released back into the wild (Conway, 1986). With these issues, the goal of continually producing healthy, genetically valuable offspring to sustain population diversity is much harder to achieve. This also does not account for logistical challenges in reproduction, such as transporting genetically valuable animals from different locations to reproduce, or the behavioral incompatibility between potential mates.

Captive populations may also face challenges arising from reduced genetic diversity in wild populations prior to captive breeding efforts beginning, undetected hybridization from founders, or studbook errors resulting in improper breeding recommendations (Witzenberger & Hochkirch, 2014). For instance, European wildcats in 30 zoos across Europe have a higher genetic diversity than the *in situ* population. However, the captive population shares mitochondrial DNA markers with domestic cats, suggesting a hybridization event that compromised the European wildcat gene pool. Although individual health may not be impacted by the introduction of domestic cat mitochondria, the wildcat's genomic DNA has been altered, rendering this population not recommended for reintroduction into their natural habitat (Witzenberger & Hochkirch, 2014). In situations like this, proactively establishing extensive gene banks can aid in preventing biodiversity loss after entire populations have vanished or been compromised. This allows researchers to save genetic material for generations to come, when assisted reproductive technologies (ART) not yet invented can help to resurrect healthy populations (Bolton et al., 2022).

No matter what technique is utilized, whether gamete cryopreservation, artificial insemination, embryo transfer, or *in vitro* fertilization (IVF), good quality, mature gametes are essential for ART success. This, by definition, excludes gametes from prepubertal animals or seasonal breeders when out of season. *In vitro* fertilization, in particular, requires careful handling for every species. Not only do each species have different responses to hormone treatments that require individualized plans for stimulation of ovarian activities, but they also need unique medium compositions that support fertilization and embryonic development (Pelican et al., 2006). However, what if a genetically valuable animal dies before they reach puberty or during non-breeding season? If the ovaries are still viable, then a method of growing and maturing gametes *in vitro* is needed. By using domestic cats as a model for wild felids, we can explore various options that will allow rescuing and utilizing genetic materials from every individual within a population.

While the main reason we are investigating domestic cats is for conservation efforts, they can also serve as a non-rodent biomedical model. In mice, successful follicular maturation and even embryo development has been accomplished, but murine follicular models do not translate well to larger mammalian species (Smitz & Cortvrindt, 2002). This is the result of anatomical differences, as well as many gaps in knowledge including *in vitro* follicle death, growth constraints, metabolic and structural requirements, and vital paracrine growth factor signals (Smitz et al., 2010; Smitz & Cortvrindt, 2002). On the other hand, cat physiology is remarkably comparable to human, including many genetic markers for neurological diseases (Lyons, 2020). Cats are also an established model for nongenetic human diseases such as asthma, HIV, obesity, and type 2 diabetes (Mukherjee et al., 2022).

Although not as similar to humans as another primate would be, cat models bypass some of the additional restrictions that working with primate systems entail. Importantly, there is essentially an unlimited supply of feline reproductive tracts, available from spay and neuter clinics. Further, cat ovaries have an abundance of different follicular developmental stages at any given time, allowing the investigations of mechanisms regulating folliculogenesis (Bristol-Gould & Woodruff, 2006). While the pattern of the feline reproductive cycle is slightly different from humans, cat oocyte morphology is similar to human. For instance, in cats, both the oocyte and germinal vesicle diameters, at 110 µm and 45 µm respectively, are the same as human (Rojo et al., 2015). As a reference, mouse oocyte and germinal vesicle diameters are 90 µm and 30 µm, respectively (Calarco et al., 1972). While mature follicle size is different between a cat and a human (4 mm and 23 mm, respectively), this is not due to any significant difference in follicle composition or structure. Rather, this is due to how much follicular fluid is present (Bächler et al., 2014). Beyond the oocyte and follicle, the feline genome also has high synteny to the human genome. Specifically, the feline genome has the most conserved chromosome segmentation as compared to the human genome, and the genome assembly is about four times more similar between humans and felids than humans and mice or rats (O'Brien et al., 2002). Feline embryos also undergo asymmetrical divisions similar to humans (Chavez et al., 2021). Therefore, by studying cat follicular development, we may be able to identify new avenues for human fertility treatments. These can then be applied in novel ways, such as the ability to generate competent oocytes from women who undergo chemotherapy and suffered fertility issues as a result.

1.2. Felid Reproduction

The reproductive cycle of domestic cats is influenced by the duration of daylight. Specifically, cats living outside in the Northern hemisphere enter anestrus between September

and January, while cats kept indoors may continually display estrus behaviors throughout the year (Jemmett & Evans, 1977). Puberty typically onsets between 5 and 9 months of age, depending on breed, nutritional state, and the time of year they were born (Pereira et al., 2010). The estrous cycle consists of four stages: proestrus, estrus, diestrus, and anestrus. Proestrus is characterized by the queen becoming more affectionate, with estradiol concentrations beginning to rise. Unlike in dogs, this phase does not differentiate itself behavior-wise very well in cats. It also lasts approximately one day. Therefore, it is easy to miss this phase (Pereira et al., 2010). Next, estrus is marked by the elevated estradiol levels. These levels allow the follicles to finish maturation and contribute to estrus behaviors. Receptivity to the tom cat, thrashing on the floor, and calling to attract attention all behaviorally indicate estrus. The queen will allow copulation at this time, which in turn will trigger ovulation. If copulation has not occurred, the queen will enter interestrus. Estrogen levels fall, and she will not be receptive to mating until estrus begins again (Pereira et al., 2010). Diestrus, or luteal phase, occurs after ovulation occurs. This stage is characterized by the rise of progesterone concentration which remain elevated for 65-67 days and 40 days in pregnant and non-pregnant female, respectively. Diestrus is followed by anestrus stage that is characterized by the period of ovarian inactivity. In cats that breed seasonally, anestrous period is influenced by decreased daylight and timings will vary based on location (Pereira et al., 2010).

1.3. Ovarian Folliculogenesis

For the general mechanics, folliculogenesis is standard across mammalian species and begins in the ovary's primordial follicle reserve (Figure 1.1). This is a large, fixed pool of follicles that are waiting for activation. In many species, including the cat, this pool is initially established in the fetus. Primordial germ cells first migrate to the genital ridge, where they

differentiate into oogonia (Peters & McNatty, 1980). The oogonia proliferate, then arrest mitosis and undergo mass apoptosis, bringing the numbers of primordial oocytes down to a fixed number. The oocytes, through the interactions of cytokines and receptors, will recruit somatic cells in the ovary to form primordial follicles (Table 1.1). However, time points for oocyte number fixation and full assembly of the primordial follicles can vary by species, ranging from just before birth to just after (Van Den Hurk & Zhao, 2005).

Primordial follicles only begin to grow once activated, and the growth is tightly regulated by many protein interactions (Table 1.2). There is no information about the time at which follicle activation begins in the cat. However, studies in other species, including the human, show that primordial follicles activate and enter the growth phase before puberty, although ovulation does not occur until puberty (Peters & McNatty, 1980). Activation of primordial follicles involves highly conserved pathways across mammalian taxa, such as phosphatidylinositol 3-kinase (PI3K)-Protein Kinase B (Akt), transmembrane receptor protein serine/threonine kinase, and transforming growth factor (TGF)-β (Kehoe et al., 2021). Primordial follicles consist of two cell types: the oocyte and the pre-granulosa cells (Field et al., 2014). At this quiescent stage, the pre-granulosa cells are flattened and consist of a single layer, while the oocyte is arrested at the diplotene stage (Monniaux, 2016; Reynaud et al., 2009).

After activation, the granulosa cells crowd together and become cuboidal. The oocyte will increase in size, with overall follicle growth linearly aligning with oocyte growth at a slope of 0.526 (Reynaud et al., 2009). This represents the follicle's transitions into first a primary follicle, then a secondary follicle, which now has multiple layers of granulosa cells. As the secondary follicle continues to develop, the theca cell layer establishes, thickens, and develops vasculature, which allows steroidogenesis to begin. After passing the secondary follicle stage,

the follicle develops a fluid filled cavity, or antrum, through protein interactions. This marks the antral phase (Table 1.3). It is at this stage that the follicle begins responding to follicle stimulating hormone (FSH), which allows the follicle to be "recruited" and continue growing. Overall follicle growth also outpaces oocyte growth, breaking the previous linear relationship in favor of a more flattened slope (slope = 0.10) (Reynaud et al., 2009). Finally, the follicle reaches the Graafian, or preovulatory, stage. The follicle wall undergoes substantial remodeling in preparation of ovulation. Luteinizing hormone (LH) from the anterior pituitary initiates meiosis in the oocyte, starting from prophase I, and arresting at metaphase II, where it will remain until fertilization (Mehlmann, 2005; Turathum et al., 2021). An LH surge triggers ovulation of the metaphase II oocyte, and cumulus cells surrounding the oocyte undergo expansion, in preparation for sperm selection and fertilization (Turathum et al., 2021). The remaining follicle cells undergo differentiation and transform into a corpus luteum (CL), secreting progesterone to support pregnancy.

Table 1.1: Primordial Follicle Formation Protein Interactions. A selection of protein interactions integral for fetal primordial follicle formation.

Protein	Expression site	Target site	Function	Species
BMP-2,4,8b (bone morphogen protein)	Endoderm and extra-embryonic ectoderm	Epiblast	Primordial germ cell formation	Mouse[1]
KITL (KIT ligand)	Somatic cells along path of migration	Primordial germ cells	Guides migration of primordial germ cells, aids in proliferation of germ cells	Mouse, human[1]
LIF (leukemia inhibiting factor)	Somatic cells along path of migration	Primordial follicles	Works with KITL to increase germ cell number	Mouse[1]
E-cadherin	Primordial germ cell	Genital ridge	Compaction and colonization of the genital ridge	Mouse[1]
NT-4 (neurotropin 4)	Oogonia	Oogonia/fetal ovary somatic cells	Formation of oogonia from primordial germ cells	Human[1]
Estradiol	Somatic cells at the cortex-medulla junction	Fetal ovary	Formation of cortical cords from sex chords	Sheep[1]
Germline-α	Oocyte	Pregranulosa cells (ovarian interstitial or surface of the ovarian epithelium)	Assemble cells into primordial follicle in utero	Mouse[2]
Notch-1	Granulosa cell	Jagged 1 receptors on granulosa cells	Assemble cells into primordial follicle in utero	Mouse[2]
Estrogen	Fetal ovary	Fetal oogonia/primordial follicle reserve	Suppression of activation (fetal block to folliculogenesis)	Bovine[2]

[1]Van Den Hurk & Zhao, 2005; [2]Binelli & Murphy, 2010

Table 1.2: Preantral Follicle Growth Protein Interactions. A selection of proteins integral for preantral follicle growth and regulation.

Protein	Expression site	Target site	Function	Species
Nobox (newborn ovary homeobox protein)	Ovary	Primordial follicles	Suppression of activation - upstream transcription factor that regulates oocyte expressed genes like *GDF9*, *BMP15*	Mouse[1]
GDF9 (growth differentiation factor 9)	Oocyte	Primordial follicles	Aids activation from primordial to primary	Mouse[1]
BMP4/7/15	Oocyte	Primordial follicles	Aid activation from primordial to primary	Mouse[1]
KITL	Granulosa cell	Primordial follicles	Aids activation from primordial to primary	Mouse[1]
AMH (anti-Mullerian hormone)	Granulosa cell	Primordial follicles	Suppression of activation	Mouse[1]
Foxl2 (forkhead box L2)	Somatic ovary cells	Primordial follicles	Aids activation - regulates downstream effects such as StAR (which in turn regulates steroidogenesis). Reverses effects of estrogen block of activation	Bovine[1]
CX43 (connexin 43)	Granulosa cell	Granulosa cell	Intracellular membrane channel to allow flow of nutrients between cells. These are expressed from the beginning of folliculogenesis, and knockout models arrest folliculogenesis at primary stage.	Mouse[2]
GDF9	Oocyte	Primary/preantral follicle	Proliferation of follicle somatic cells, follicle growth	Mouse[1]

Inhibin	Granulosa cell	Primordial to antral follicles	Endocrine control of FSH	Cat[3]
Activin	Granulosa cell	Primordial to antral follicles	Paracrine signals for follicular growth	Cat[3]
EGF (epidermal growth factor)	Granulosa cell of primary follicle	Ovarian somatic cells surrounding the follicle	Recruitment of somatic cells to differentiate into theca cells for primary to secondary follicle transition.	Mouse[2]
TGFβ (transforming growth factor beta)	Granulosa cell	Preantral follicle	Preantral follicle growth	Sheep[1]
ALK3/5/6 (activin-like receptor kinases 3, 5, 6)	Granulosa cell	Preantral follicle	Preantral follicle growth	Sheep[1]
GH (growth hormone)	Pituitary gland	Preantral follicle	Preantral follicle survival	Mouse[1]

[1] Binelli & Murphy, 2010; [2] Gougeon, 2010; [3] Bristol & Woodruff, 2004

Table 1.3: Antral Follicle Growth, Ovulation Regulation, and Corpus Luteum Maintenance Protein Interactions. A selection of proteins integral for regulation and growth of antral follicles, ovulation, and the corpus luteum.

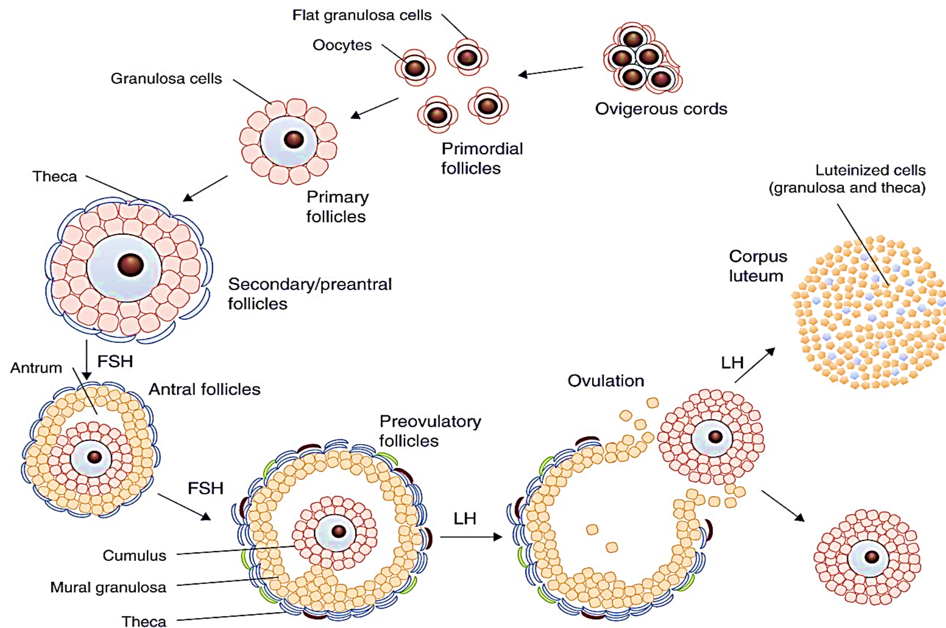
Protein	Expression site	Target site	Function	Species
γ-glutamyl transpeptidase	Somatic cells of follicle	Preantral follicle	Involved in glutathione metabolism. Allows for transition to antral follicle and continuation of folliculogenesis	Mouse[1]
Aquaporin 7/8	Granulosa cell (membrane protein)	Granulosa cells/antral follicles	Antrum formation - allows movement of fluids and serum around theca cells into the interior of the follicle, forming the antrum	Bovine[2]
FGF8 (fibroblast growth factor)	Oocyte	Antral follicle/granulosa cells	Antrum formation and regulation	Mouse, rat[2]
FSH	Pituitary gland	Antral follicle	Antrum formation and growth	Bovine[2]
FSHR (FSH receptor)	Granulosa cells	Early antral stage follicles	Receptor for FSH, allows for early antral follicle and antral follicle growth. Atretic follicles decrease expressions of FSHR	Cat[3]
CYP19 (aromatase)	Granulosa cell	Granulosa cell	Production of estrogens in antral follicles	Mouse[2]
Hyaluronan, versican and inter-α trypsin inhibitor (glycosaminoglycans)	Follicular fluid	Antral/preovulatory follicles	Osmotically active, helps to grow antrum to preovulatory size. Also helps cumulus expansion after ovulation	Bovine[2]
GDF9, TGFβ, activin-A	Oocyte	Granulosa cell	Proliferation of granulosa cells	Mouse[2]

GDF9	Oocyte	Granulosa cell	Morphology changes towards preovulation granulosa cell phenotypes, transition to cumulus cells	Mouse[2]
GDF9, BMP15, FGF8	Oocyte	Cumulus cells	Maintain differentiation from granulosa cells, anti-apoptosis factors	Mouse, bovine[2]
IGF1 (insulin-like growth factor 1)	Theca cell	Granulosa cell	Granulosa cell proliferation, steroidogenesis in conjunction with gonadotropins	Rat, pig[2]
FGF2,7	Theca cell	Theca cells of antral follicles	Follicle growth	Bovine[2]
LH (luteinizing hormone)	Pituitary gland	Antral follicle	Pulsatile action allows dominant follicles to grow, once they have expressed LH receptors. Triggers ovulation	Bovine[2]
LHR (luteinizing hormone receptor)	Granulosa cell	Antral follicle	Development allows selection of dominant follicle in monovulatory species. Enhances CYP19 expression, increases secretions of estrogens	Bovine[2]
LHR	Theca cell (early antral)	Antral follicle	Stimulates steroidogenesis in theca cells (androgenic steroids). This is discontinuous, meaning that not every theca cell expresses LHR and therefore not every theca cell conducts the thecal half of steroidogenesis.	Cat[3]

PKA (protein kinase A) pathway	Somatic cells of follicle	Somatic cells of follicle	Intracellular pathway that responds to LH surge and results in ovulation. Downstream effects include EGF (epidermal growth factor) family regulation of cumulus expansion and meiotic resumption of oocyte	Mouse[2]
LHR	Corpus luteum	Corpus luteum	Maintenance of corpus luteum	Cat[3]

[1]Gougeon, 2010; [2]Binelli & Murphy, 2010; [3]Saint-Dizier et al., 2007

Figure 1.1: Folliculogenesis. A diagram depicting the folliculogenesis cycle (Georges et al., 2014). The ovigerous cords form in the fetal ovary, and develop into primordial follicles, with an oocyte and flat granulosa cells. After activation, primordial follicles become primary follicles. Theca cells are recruited, marking a secondary follicle. Responsivity to FSH allows the follicle to develop an antrum, becoming an antral follicle, then a preovulatory follicle. A LH surge triggers ovulation of the cumulus-oocyte complex, while cells left in the ovary undergo reorganization to become a corpus luteum.



The activation of primordial follicles is regulated by a milieu of cytokines and growth factors. Studies in the mouse have shown that KIT ligand (KITL) secreted from granulosa cells activates PI3K and its downstream target the mammalian target of rapamycin (mTOR) in the oocyte. mTOR is directly responsible for the follicle activation by enhancing glycolysis and proliferation of the granulosa cells (Monniaux, 2016; Zhang et al., 2022). A mouse study has shown that overexpression of mTOR complex 1 (mTORC1) results in activation of the entire reserve of primordial follicles, causing premature follicle depletion (Monniaux, 2016). Other growth factors like basic fibroblast growth factor (FGF2) and leukemia inhibitory factor (LIF), secreted by the granulosa cells also has been shown to play roles in primordial follicle activation (Field et al., 2014). Meanwhile, the ovarian stroma, some of which will be recruited as theca

cells, are producing bone morphogenetic proteins (BMP), specifically BMP4 and BMP7. These morphogens target the SMAD pathway, and in turn regulate the expression of several nuclear transcription cofactors (Field et al., 2014). As a result, the granulosa cells begin to mature, expressing more KITL for the oocyte's continued growth.

Like almost all biological processes, recruitment of follicles and subsequent growth requires a delicate balance. Inhibitory factors are as equally important for follicular development as the activating factors. One major inhibitory factor is anti-Mullerian hormone (AMH). In male fetuses, AMH is responsible for the degradation of the Mullerian ducts, which would form the female reproductive tract. However, in post-pubertal females, AMH acts as a suppressant to follicular recruitment. This maintains the primordial follicle pool over the course of a female's reproductive lifetime (Field et al., 2014).

Primary follicle development is controlled by paracrine and autocrine factors from the oocyte, granulosa cells, and ovarian stroma. One of the TGFβ family molecules released from the oocyte is the growth differentiation factor 9 (GDF9), an obligatory morphogen for continued folliculogenesis. GDF9 has many roles throughout folliculogenesis, including activating steroidogenesis in the follicle by upregulating cyclooxygenase 2 (COX2), hyaluronan synthase 2 (HAS2), and steroidogenic acute regulatory protein (StAR), essential proteins for steroid hormone production (Field et al., 2014). Without GDF9, follicles can reach the primary stage. However, to move to the secondary follicle stage, GDF9 is required. In GDF9 knockout mouse models, oocytes increase in size abnormally by up to 70% and reduce production of cortical granules. Granulosa cell proliferation also decreases, and concurrently overexpress KITL and under express FSH receptors. Meanwhile, theca cells do not proliferate (Erickson & Shimasaki, 2000). Less is known about somatic cell contributions to primary follicle growth (Binelli &

Murphy, 2010). However, granulosa cell produced AMH may play a role in preventing over-proliferation of granulosa cells in mice (Binelli & Murphy, 2010).

The transition from primary to secondary stage is marked by granulosa cell proliferation. At this point, the oocyte is surrounded by multiple layers of granulosa cells and components of the zona pellucida start to be deposited around the oocyte. Theca cells also develop and proliferate, forming new vasculature via angiogenesis to help oxygenate the more metabolically active follicle (Aerts & Bols, 2010). Insulin-like growth factor (IGF)-1 and -2 are expressed from the theca cells, and stimulate secondary follicle growth (Binelli & Murphy, 2010). In short, primary follicles have about 50 somatic cells around the oocyte, while secondary follicles are approaching 200 somatic cells (Kehoe et al., 2021). While primordial follicles mainly reside on the outer edges of the cortex, primary and secondary follicles begin moving towards the less rigid, more oxygenated medulla to allow for better growth and exchange of nutrients. However, the transition from primary to secondary follicle is not well understood in cats. A recent study investigated differential gene expression among primordial, primary, and secondary follicles in the domestic cat. At the transition from primary to secondary, the follicles showed an upregulation of epidermal growth factor (EGF), which is known to upregulate the PI3K pathway (Kehoe et al., 2021). In mice, this cascade, in conjunction with matrix metalloproteinases, has been shown to play a role in the movement of follicles away from the outer edges of the cortex (Kehoe et al., 2021). In cats, EGF is essential for maintaining primordial follicle viability before activation, by promoting ovarian cell proliferation through the mitogen active protein kinase (MAPK) and PI3K pathways (Fujihara et al., 2014). EGF also plays an active role for angiogenesis in secondary follicles (Fujihara et al., 2014). Combined with FGFs and vascular

endothelial growth factor (VEGF), EGF activates theca cell proliferation via the MAPK pathway (Field et al., 2014).

The tertiary stage, also known as the antral phase, can be subdivided into two parts – early antral follicles and preovulatory (Graafian) follicles. It is at this point that follicles switch from gonadotropin-independent to gonadotropin-dependent for continued growth. However, in cats, little is known about the exact mechanism for how this switch occurs. FSH is critical for follicle development by stimulating granulosa cell proliferation, antral formation, and steroidogenesis (Webb et al., 1999). To properly guide development, FSH receptors are highly localized in granulosa cells in the cat antral follicle, while LH receptors are present in antral follicle theca cells and large antral follicle granulosa cells (Saint-Dizier et al., 2007). Paracrine factors, including BMP-15 and GDF9 produced by the oocytes also play roles in regulating follicle development. Although both BMP-15 and GDF9 promote granulosa cell proliferation, BMP-15 also regulates follicle selection by down-regulating FSH receptor expression that in turns inhibits the stimulating effect of FSH on the follicle (Field et al., 2014). Other factors, including IGF1, activin, and inhibin also have been shown to play roles in follicle development by regulating proliferation of granulosa cells, theca cells, and steroidogenesis (Namwanje & Brown, 2016).

Ovulation occurs when estradiol levels peak, triggering a subsequent surge of LH. However, this surge only happens after appropriate stimulation via copulation in the domestic cat. While there are reports of spontaneous ovulation in unmated queens, there is little known about exact mechanisms. Available evidence does suggest that this happens most frequently in virgin, older queens, although why and how remains to be examined (Pereira et al., 2010). The released oocytes have a network of granulosa cells surrounding them, forming the cumulus

oocyte complex (COC). The remaining granulosa and theca cells undergo conformational changes, compacting down into a denser, polyhedral structure surrounded by blood vessels to form the corpus luteum (CL). The CL reaches full maturation and size 12-16 days after ovulation (Jewgenow et al., 2012). This allows for adequate production of progesterone for the maintenance of pregnancy. If pregnancy is not established, CL regression occurs when the cells lose their ability to produce progesterone and levels subsequently drop. In pseudopregnant queens, this process begins around day 21, while in pregnant queens, the CL will remain until after lactation finishes (Jewgenow et al., 2012). This regression allows for new follicles to fully mature, restarting the cyclical process of folliculogenesis.

Ovarian folliculogenesis is further regulated by several endocrine factors. Hormonally, this begins with gonadotropin releasing hormone (GnRH). As seasonal breeders, cats only begin producing this hormone during the spring, when melatonin, which acts as a GnRH suppressant, decreases due to increasing daylight lengths (Senger, 1999). GnRH then acts on the pituitary, which in turn releases FSH and LH. FSH levels rise much faster than LH, as they are crucial to the recruitment and development of early stage to preovulatory follicles. As follicles grow, granulosa cells produce estrogens, which act as a positive feedback loop for continued gonadotropin release. In domestic cats, estrogen levels vary during estrus, but are generally above 20 pg/mL (Wildt et al., 1981). Mating triggers a surge of LH levels from about 4 ng/mL to over 70 ng/mL, which results in ovulation and marks the luteal phase (Pohler et al., 2020; Wildt et al., 1981). The progesterone secreted by the maintained CL and later the placenta prevents estrus from restarting. Prostaglandin F2α (PGF2α) is a luteolytic factor that degrades the CL to prevent further progesterone secretion in most mammals, including the majority of felids (Amelkina et al., 2015; Jewgenow et al., 2012). The exception in felids is the lynx, where

progesterone production is only slightly decreased after PGF2α treatment, and the CL structure remains on the ovary. To date, the exact mechanisms for persistent corpora lutea in lynx has not been fully elucidated. Nevertheless, studies have indicated that other apoptotic factors, such as BCL2-associated X protein (BAX), caspase-3 (CASP3), and tumor necrosis factor (TNF) may have a role in luteolysis in this species (Amelkina et al., 2015).

1.4. *In vitro culture of isolated follicles*

While many species have been utilized for *in vitro* follicular development studies, rodents remain the vast majority, with 44% of papers utilizing rodent models. Humans and non-human primates come in second and third, at 16% and 15%, respectively (Simon et al., 2020). Other common species used are agricultural animals, including bovine (9.1%), ovine (4.6%), caprine (4.6%), and porcine (2.3%). Cat studies comprised 2.3% (Simon et al., 2020).

1.4.1. Recovery of ovarian follicles for *in vitro* culture

There are two main methods to remove developing follicles from surrounding ovarian tissue: mechanical isolation and enzyme digestion (Simon et al., 2020). Mechanical isolation relies on a technician cutting the follicles free from the surrounding cortex, usually with a pair of needles that range in size from 25 gauge to 31 gauge or by passing the tissue through a dissection sieve (Simon et al., 2020). This method has been used in bovine, caprine, human, murine, ovine, porcine, nonhuman primate, and feline follicles, usually at the secondary, preantral, and antral stages (Abir et al., 1997; Baba et al., 2017; Craig et al., 2010; Ferreira et al., 2016; Gutierrez et al., 2000; Itoh et al., 2002; Jin et al., 2010; McLaughlin et al., 2014; Nagashima et al., 2021; Nation & Selwood, 2009; Peluffo et al., 2013; Rossetto et al., 2013; Thomas et al., 2001; Thongkittidilok et al., 2018; Wu et al., 2001). Enzymatic preparations, e.g., Liberase™,

collagenase, or a combination of the two, have been used to recover primordial to secondary follicles in bovine, canine, feline, human, murine, and ovine models (Desai et al., 2012; Diaz et al., 2007; Laronda et al., 2014; Muruvi et al., 2005; Nagashima et al., 2021; Songsasen et al., 2011; Wandji et al., 1996; Yuan & Giudice, 1999).

Each of the isolation methods have advantages and disadvantages. Mechanical isolation easily preserves three dimensional structures of the follicle. However, mechanical methods also require training and are time consuming, which means that successful follicle isolation depends on user ability and dexterity (Araújo et al., 2014; Nagashima et al., 2021). This naturally introduces variation into a data set, which is where enzymatic isolation may be preferred. Furthermore, enzymatic isolation does not require visualization of follicles, as microdissection with needles does, meaning that smaller follicles at the primordial or primary stage can be easily obtained. Yet, the enzymes can degrade cellular connections within the follicle, i.e., between the granulosa cells and theca cells, which in turn damages the basement membrane (Demeestere et al., 2002; Telfer, 1996). Therefore, selection of a method may depend on study goals and desired initial follicle stage, not necessarily species.

1.4.2. *In vitro* follicle culture

To date, *in vitro* follicle culture studies have employed either two-dimensional (2D) or three-dimensional (3D) culture systems. Two-dimensional cultures involve culturing isolated follicles in a culture dish or plate without a supporting matrix surrounding the follicle. Live mice have been produced from 2D *in vitro*-grown follicles (Eppig & O'Brien, 1996). This culture system also has been used to culture isolated follicles in other species, resulting in production of antral follicles in rhesus macaque (J. Xu et al., 2018) and cattle models (Gutierrez et al., 2000). Yet despite these successes, a 2D culture system does not support integral three-dimensional

follicular structure and the essential connection between follicle cells and the oocyte. This makes it a less than ideal culture system.

Three-dimensional cultures, on the other hand, offer a much more promising path forward for follicular cultures, not only for mice, but also for other species (Simon et al., 2020). These cultures can take a variety of forms, but most techniques encapsulate a developing follicle within a biomaterial to support the spherical structure and the communication between the granulosa cells and enclosed oocyte. The culture can also be maintained for extended periods, to allow follicles more time to develop (West et al., 2007). This is especially useful for large animal species that take weeks or months to complete folliculogenesis. Direct comparisons of 2D cultures to 3D cultures in the goat and mouse found 3D cultures result in higher follicular survival, lowered premature oocyte extrusion, and increased mRNA expression of follicle health markers, including *GDF9*, *BMP15,* and *BMP6* (Sadr et al., 2015; Silva et al., 2015).

Alginate, fibrin, Matrigel™, and collagen have all been previously investigated for their ability to strike a balance between offering support to the cellular structures while still allowing room for growth (Khunmanee & Park, 2022). In particular, alginate has been used successfully in producing developmentally competent oocytes in mice, goats, and humans (Brito et al., 2014; Xiao et al., 2015; M. Xu, Banc, et al., 2009). Live mice have also been produced from alginate cultured follicles (M. Xu et al., 2006). Studies have shown that optimal concentrations of alginate vary among species. While the alginate must be strong enough to support follicular architecture, high concentrations can result in a rigid environment that restricts expansion (Vanacker & Amorim, 2017). Mouse secondary follicles grew larger and formed an antrum at higher rates in a permissive microenvironment of 0.5% alginate than those incubated in 1.5% alginate (West-Farrell et al., 2009). Goat preantral follicles cultured in higher concentrations of

0.5% - 1% have better overall structure, but lowered hormonal production compared to follicles encapsulated in 0.25% alginate (Brito et al., 2014). In cats, 0.3% - 0.5% alginate supplemented with FSH supported early antral and antral follicular growth and structural development (Nagashima et al., 2021; Songsasen et al., 2012). Additionally, 0.5% alginate showed a significant increase in viability for the COCs recovered from incubated follicles compared to a 2D culture (Morselli et al., 2017). However, there are no studies evaluating the impact of alginate concentration on the development of varying stage cat follicles.

Previous studies have indicated that there are stage specific differences in alginate concentration that support the survival of incubated follicles. Specifically, primate primordial follicles required a rigid microenvironment of 2% alginate to maintain structural integrity and growth *in vitro* (Hornick et al., 2012). However, primary and secondary primate follicles encapsulated in permissive biomaterials, a fibrin (50 mg/ml)-alginate (0.5%)-Matrigel^{TM} matrix, are able to develop and produce meiotically competent oocytes (M. Xu et al., 2011). Further, a study in the mouse has demonstrated differences in maturation between two-layer and multi-layered secondary follicles to alginate that has been modified with various types of additional extracellular matrices (Kreeger et al., 2006). These differences can be explained by opposing needs for support versus room for expansion. As primordial follicles do not have the same structural support from developed granulosa cells, they need more rigid support from the alginate. Further, as described above, primordial follicles are located primarily in the outer edges of the cortex, which is structurally more rigid than regions closer to the medulla. Yet as follicles grow, the extracellular matrix must be flexible enough to accommodate that growth, which necessitates lower alginate concentrations. Unfortunately, alginate at a fixed concentration does not biodegrade or remodel (Vanacker & Amorim, 2017). When adding a biodegradable material,

fibrin, to the alginate, though, mouse follicles had lower mechanical stress and harvested oocytes showed an increased ability to resume meiosis (Shikanov et al., 2009). This shows that the rigidity of the culture microenvironment is highly important for follicular development (Woodruff & Shea, 2011).

1.5. Co-cultures

In vitro maturation of primordial follicles has resulted in the production of mature oocytes in the mouse (Eppig & O'Brien, 1996). However, replicating the complex *in vivo* conditions that relies on the intricate relationships between hormones and signaling ligands can be challenging. One way to help overcome this shortfall is to co-culture follicles with another cell type, such as adipose-derived stem cells, mouse embryonic fibroblasts, ovarian stromal cells, theca cells, and endothelial cells (Green et al., 2019; Kedem et al., 2017; Tagler et al., 2014; Tingen et al., 2011). These co-culture cells secrete growth factors and cytokines such as VEGF, FGF2, IGF-1, and EGF, which have been shown to support follicle and oocyte development as well as hormone production (Lee, 2020). Species studied for follicular co-culture include the mouse, goat, human, and cow (Derrar et al., 2000; Hosseini et al., 2020; Kedem et al., 2017; C.-H. Kim et al., 2013; Qiu et al., 2014).

The ovarian stroma is comprised of a wide variety of different cell types and structures, including neurons, blood vessels, immune cells, ovarian surface epithelium, tunica albuginea, fibroblast-like cells, and interstitial cells (Kinnear et al., 2020). Among these cells, endothelial cells are the most characterized. These large and diverse populations of cells work together by secreting cytokines, growth factors, morphogens, and extracellular matrix proteins to support the follicular development and ovarian function (Kinnear et al., 2020). While the majority of cells in

the ovarian stroma are poorly characterized, their functions might vary based on location, age, and timepoint in estrus cycle (Kinnear et al., 2020).

Mesenchyme-derived endothelial cells line the inside lumen of blood vessels (Korff & Augustin, 1998). Therefore, they can be purified from almost any tissue of the body. Endothelial cells are characterized by the expression of cell surface markers CD-31 and CD-34, and distinguishable from blood cells by the lack of CD-45 (Crouch & Doetsch, 2018). Investigations have detected endothelial cells not only in blood vessels, but also around the ovarian stroma surrounding primary and early antral follicles and in the thecal cell layer around late preantral to early ovulatory follicles (Kizuka-Shibuya et al., 2014). In fact, endothelial cells induce organogenesis before blood circulation fully develops in an embryo. They secrete so-called angiocrine factors, including Jagged 1, VEGF, and platelet-derived growth factor-β (PDGFβ) that play crucial roles in promoting organ patterning, angiogenesis, tissue repair, and self-renewal of hemopoietic and neural stem cells (Butler et al., 2010; Kedem et al., 2017). Within the ovary, endothelial cells express VEGF receptors and FGF-2 to regulate angiogenesis and ovarian follicle development (Bruno et al., 2009). These regulatory signals play a vital role in providing nutrients and oxygen to developing follicles.

1.6. Conclusion

Folliculogenesis *in vitro* has much to be examined, especially in cats. From understanding the exact mechanisms of how feline follicles mature, to the best culture conditions to encourage this growth, this complex physiological process is crucial to conservation efforts and fertility treatments. While there have been some studies investigating co-cultures of felid oocytes for *in vitro* maturation, none specifically investigated co-culture's effect on follicle maturation (Morselli et al., 2017). Therefore, the objectives of my research were to: 1)

investigate ovarian stromal cell coculture effects on preantral, early antral, and antral feline follicles; 2) identify if coculture cells must be present, or if conditioned media at varying concentrations would produce similar effects; and 3) confirm follicle function and oocyte competence via expression of a selected suite of follicular health markers (*GDF9, FSHR, CYP19A*) and meiotic resumption.

Chapter 2: Ovarian Stromal Cell and Follicular Co-culture

2.1. Introduction

Within the mammalian ovary, thousands of primordial follicles are embedded in the cortex (Senger, 1999). Because of mechanisms that have not been fully characterized, primordial follicles activate and develop into primary, secondary, and antral follicles. However, only a few of these follicles will fully mature and ovulate a developmentally competent oocyte, leaving an untapped wealth of unused germplasm (Senger, 1999). Replicating folliculogenesis *in vitro* is key to fertility preservation, which can be applied to endangered wildlife and women affected by infertility (Pelican et al., 2006). However, challenges to complete this process remain, especially in large, non-rodent models (Simon et al., 2020).

The domestic cat is a midsized mammalian model, which makes it ideal for human focused ovarian research. It also serves as a model for endangered feline species (Swanson, 2003). To date, *in vitro* culture of isolated follicles involves incubating a follicle in a two or three-dimensional culture system (Simon et al., 2020). In the cat, specifically, developmentally competent oocytes have been collected from *in vitro* cultured antral follicles encapsulated in alginate (Songsasen et al., 2012). However, this success has not been replicated in primordial or primary feline follicles.

One aspect limiting *in vitro* folliculogenesis success may be the lack of cross communication between isolated follicles and somatic cells present in the ovary, including ovarian stromal cells (Green & Shikanov, 2016). Therefore, introducing a secondary cell line via co-culture may restore missing paracrine factors. Studies conducted in the mouse and buffalo demonstrate beneficial effect of co-culture systems on ovarian follicle development (Green &

Shikanov, 2016). Past examples of co-culture cell lines include adipose-derived stem cells, mouse embryonic fibroblasts, ovarian stromal cells, theca cells, or endothelial cells (Green et al., 2019; Kedem et al., 2017; Tagler et al., 2014; Tingen et al., 2011). Another alternative way to restore the missing paracrine signals but without introducing a potentially confounding factor, such as overgrowth, is incubating follicles in conditioned medium obtained from the cultured cell lines (H.-E. Hong et al., 2019; Ra et al., 2023). Culturing mouse preantral follicles in endometrial cell conditioned media improved preantral follicle development and increased developmental competence of *in vitro* grown oocytes by maintaining granulosa cell viability and function (Taghizabet et al., 2022). Further, conditioned medium of human umbilical cord mesenchymal stem cells has been shown to improve maturation and protect against ovarian damage in the mouse by stimulating the PI3K-Akt pathway (L. Hong et al., 2020).

The few studies in feline folliculogenesis that have investigated co-cultures looked at the impact on oocytes, embryos, and ovarian cortex strips (Baouche et al., 2023; Vilela et al., 2016). Oocytes matured with mesenchymal stem cell co-culture did not show increased rates of meiotic resumption, but embryos cultured with the same cell population showed higher rates of cleavage, and higher rates of morula and blastocyst formation (Baouche et al., 2023). Primordial follicle numbers in feline ovarian cortex strips cultured in chick embryo chorioallantoic membranes were significantly lower than cortex strips maintained in a static well plate, but vascularization in the tissue improved (Vilela et al., 2016). Yet to date, no research has been conducted with isolated cat follicles and co-cultures or conditioned media in the domestic cat.

The objective of the present study was to determine the influence of ovarian stromal cell co-culture and conditioned medium on feline *in vitro* folliculogenesis and mRNA expression of three follicular health genes: *FSHR*, *GDF9*, and *CYP19A*. I hypothesized that the presence of

ovarian stromal cells or conditioned medium would affect the growth and survival of preantral, early antral, and antral cat follicles.

2.2. Materials and Methods

All chemicals were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

2.2.1. Ovarian stromal cell isolation and culture

Cat ovarian stromal cells were enzymatically isolated using a modified method described previously(Soares et al., 2015). Briefly, ovarian medullas (n = 3 cats) were finely minced, then placed in a digestive medium of phosphate buffered saline (PBS), collagenase (0.5 mg/mL), and DNase I (0.01 mg/mL). The tissue was rocked at 37°C for 30 minutes, then the medium strained sequentially through a 100 µm filter and a 40 µm filter. The collected medium was transferred into a 15 ml conical tube and an equal volume of PBS + 10% fetal bovine serum (FBS) was added to the tube. The tissue pieces were placed back in fresh digestive medium and incubated for an additional 30 minutes until the tissue sufficiently disassociated, then strained again. The collected medium and an equal volume of PBS + 10% fetal bovine serum (FBS) was added to the conical tube. The medium was centrifuged at 1000 RPM for 5 minutes, then the pellet washed with 1X RBC lysis buffer, then PBS + 1% bovine serum albumin (BSA), then PBS + 0.04% BSA. Cells were cultured in endothelial cell growth medium (EGM) (PromoCell, Heidelberg, Germany) + 10% FBS at 38°C in humidified 5% CO_2 until 70-80% confluent. Cells were passaged twice - first to a T25 flask, then to a T75 flask. After the second passage achieved confluency, cells were cryopreserved and stored in liquid nitrogen until use.

2.2.2. Cryopreservation, recovery, co-culture of ovarian stromal cells

Cells (2nd passage) at a concentration of 2×10^6 cells/mL were suspended in EGM supplemented with 10% FBS and 10% dimethyl sulfoxide. Suspended cells (1 ml) were placed into cryotubes and slow frozen overnight at -80°C. The next day, the cryotubes were plunged into liquid nitrogen for long term storage. Cells were recovered by placing the tubes in a warm water bath 37°C for 2 minutes, then contents were dropwise diluted in 9 mL EGM in a 15 ml conical tube. The tube was centrifuged at 1000 RPM for 5 min and the supernatant discarded. The cell pellet was resuspended in EGM and cultured in a T25 flask at 38.5°C in 5% $CO2$ until 80% confluent. At that point, the cells were passaged and cultured in EGM (-) (EGM + 2% FBS, without VEGF, IGF, and FGF supplementation). After 24-48 hours, the conditioned medium (CM) was collected from the cell culture and frozen at -80°C. The cells were then passaged and seeded into a 4 well dish at 10,000 cells/well for the co-culture treatment group. All co-culture cells were allowed to establish and grow for 24 hours before follicular culture began. Remaining cells were cultured separately in a T75 until enough CM was produced for that given replicate, then discontinued.

2.2.3. Follicle isolation and encapsulation

Approval from an Institutional Animal Care and Use Committee was not required as domestic cat ovaries were obtained from local veterinarians following routine owner requested ovariohysterectomies. These are exempt studies under Title 9, Code of Federal Regulations, Subchapter A- Animal Welfare, Part 1 to 3.

Ovaries were collected from domestic cats older than 6 months old. The ovaries were placed in transportation medium (L-15 medium supplemented with 30 µg/mL penicillin G and

streptomycin sulfate, and 8.8 μg/mL ascorbic acid) and kept at 4°C until processing. Ovaries with corpora lutea were discarded. All ovaries collected in a single day were pooled.

Follicular isolation was conducted within 6 hours of surgery. All isolation procedures were performed in collection medium (Minimum Essential Medium [MEM] supplemented with 2 mM L-glutamine, 50 U/mL penicillin G, 50 μg/mL streptomycin sulfate, 0.1 mg/mL ascorbic acid, and 3 mg/mL BSA) on 37°C warming plates. The ovarian cortex was thinly sliced from the medulla and washed in collection medium. Subsequently, follicles were micro-dissected from the surrounding cortex using a scalpel and 25-gauge needles under a dissection microscope. The follicles were encapsulated in 0.5% alginate as previously described (M. Xu, West-Farrell, et al., 2009). Briefly, 2-3 follicles were washed in 100 μl alginate, then transferred to another drop of 100 μl alginate. Follicles were pulled up into a pipette, along with 4 μL of alginate, then dropped into a calcium salt solution (140 mM NaCl, 50 mM $CaCl_2$) for two minutes to crosslink. Encapsulated follicles were transferred individually to 24 well plates or pre-seeded 4 well co-culture plates, placed in 500 μL EGM (-), and supplemented with 1 μg/mL FSH. In each culture experiment (10 replicates performed on different days), follicles were distributed randomly across treatment groups, with a minimum of two follicles per treatment group.

On three collection days for the experimental cultures, fresh follicles of approximately 600-1000 μm were also isolated (n = 10 follicles), flash frozen, and stored at -80°C for the subsequent qRT-PCR analysis.

2.2.4. Co-culture and CM treatment

Follicles (120 μm to 750 μm in initial diameter, n = 155 follicles, 23 cats) were collected and classified into three developmental stages based on their average initial diameter (ID):

preantral ID ≤ 300μm (n = 83); early antral 300μm $<$ ID ≤ 500μm (n=55); and antral ID $>$ 500μm (n = 17) (Table 2.1). The follicles were randomly divided into five treatment groups: 1) EGM (-) (control, n = 31); 2) co-culture (EGM (-) + ovarian stromal cells; n = 30); 3) 20% CM, (80:20, EGM (-):CM; n = 31); 4) 50% CM (50:50, EGM (-):CM; n=31); 5) 100% CM (100% CM; n = 32).

Growth was analyzed using ImageJ. Minimum and maximum follicle diameter was measured on days 0, 4, 6, 8, 11, and 13. The two diameters were then averaged. The following formula was used to calculate percent growth each day:

$$Growth = \frac{Day\ Average\ Diameter\ - ID}{ID} \times 100$$

Positive growth was defined as greater than 5% average diameter expansion. Average diameter changes between -5% and 5% was determined to be static. Average diameter changes less than -5% for two consecutive time points indicated follicular death at the first time point.

All follicles were maintained in culture until Day 13, regardless of survival status. Co-culture cells were also photographed during follicle diameter assessment, to monitor proliferation, morphology, and health. On Day 13, follicles were removed from culture and flash frozen, for qRT-PCR analysis.

2.2.5. RNA isolation, cDNA production, qRT-PCR analysis

RNA isolation was performed using RNeasy kit (74004, Qiagen, Hilden, Germany). For RNA extraction, 100 µL of Buffer RLT were added to each frozen tube, and the follicles homogenized via mortar and pestle. Follicles from each treatment across multiples days were combined in a 1.5 mL Eppendorf tube to achieve 10-12 follicles per tube. This was defined as a technical replicate. Additional buffer RLT was then added to each Eppendorf tube to bring the final volume to 600 µL. The samples were homogenized again, before proceeding according to

manufacturer instructions. There were three qRT-PCR technical replicates for all treatment groups, with the exception of 20% CM, which had only two replicates. This was due to a replicate's low RNA concentration (2.5 ng/μL), which fell below the minimum required level for cDNA production.

Table 2.1: Follicle Distribution Among Initial Classifications and Treatment Groups.

Treatment	Antral	Early Antral	Preantral	Total
Control	4	13	15	32
Co-culture	6	8	16	30
20% CM	3	14	14	31
50% CM	2	10	18	30
100% CM	2	10	20	32

Table 2.2: qRT-PCR Primer Information.

Gene name	Forward sequence (5' to 3')	Melting temperature	Reverse sequence (5' to 3')	Melting temperature	Product length (nt)	Accession number	Source
Beta actin	ATC CAC GAG ACC ACC TTC	54°C	CAC CGT GTT AGC GTA GAG	52.5°C	75	AB051104.1	Thongkittidilok et al., 2018
FSHR	GGA TCT TTG CTT TCA TGG TC	51.6°C	AAC ATA GAG CTG TGA CAA GG	52.1°C	113	NM_001048014.1	This study
GDF9	CAT CCG TGG ACC TGC TAT TT	54.8°C	CCA GGT TGC ACA CAC ATT TC	54.8°C	129	NM_001165900.1	Chansaenroj et al., 2019
CYP19A	CAA TCC TGC TGC TCA CTG	53.6°C	CCA TGC AAT AGC CAG GAC	53.5°C	84	GU306147.1	Thongkittidilok et al., 2018

cDNA was synthesized from 50 ng RNA using iScript cDNA Synthesis Kit (1708890, BioRad, California, USA) according to manufacturer instructions. Four genes were analyzed: *Beta actin* (reference gene), *FSHR*, *CYP19A*, and *GDF9*. All reactions were performed in duplicate on a Roche LightCycler (Basel, Switzerland), with the manufacturer's SYBER green I Master Mix (04707516001, Roche, Basel, Switzerland). The following settings were used for the qRT-PCR amplification: 300 seconds (s) preincubation at 95°C, 45 cycles amplification at 95°C 15s, 53°C for 30s, 72°C for 30s, and melting at 95°C 10s, 65°C 60s, and 97°C 1s. Primer sequences, optimal melting temperature, and product length are in Table 2.2. Standard curves were separately generated for the primers via serial dilutions of cDNA to analyze primer efficiency, as described by the Pfaffl method (Pfaffl, 2001). To obtain relative expression, the experimental treatments were normalized to the cultured control follicles.

2.2.6. Oocyte Maturation

To assess development of *in vitro* grown oocytes, additional follicles (n = 199 follicles, 26 cats, 11 replicates) were isolated and cultured for 10 days in one of the following conditions: control, co-culture, or 100% CM. At the end of the incubation, 30 µL of 30 µg/mL alginate lyase (A1603) was added to each culture well, and follicles incubated for 45 min at 38°C until the alginate bead had dissolved. Follicles were collected and washed twice in follicle collection medium. Oocytes (n = 132) were released by breaking the follicle wall with a 25-gauge needle. The oocytes from each culture group were washed once in oocyte maturation medium, comprised of Quinn's Advantage™ Blastocyst Medium (ART-1029, Cooper Surgical, Connecticut, USA), 5% FBS, 10 µg/mL FSH, 1 µg/mL LH. Oocytes were transferred to an oocyte maturation medium droplet under mineral oil, the volume of which was adjusted to 10 µL

per oocyte. The oocytes were cultured 24 hours at 38°C and 5% CO_2. After culture, the oocytes were collected, washed twice in PBS, and placed in PBS + 0.2% sodium azide, 2% normal goat serum, 1% BSA, 0.1 M glycine, and 0.1% Triton X-100(Barrett & Albertini, 2007). The cumulus cells were removed by repeated pipetting. The oocytes were washed once more in PBS, before being fixed in 4% paraformaldehyde for 24 hours. Finally, the oocytes were stained with 10 µg/mL DAPI, and mounted with ProLong Gold antifade reagent (2406594, Invitrogen, Massachusetts, USA). Meiotic status of the oocyte was then classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase II (MII), or degenerated (D) (Van Blerkom & Runner, 1984).

2.2.7. Glucose testing

To determine whether ovarian stromal cells utilized significant amount of glucose, CM glucose concentration was assessed by using a BRS GM-100 (BioReactor Sciences, Georgia, USA). A glucose standard curve was generated from EGM (-). The glucose reader was keyed to MEM composition, and the standard curve data was sent back to the company to obtain a concentration correction worksheet specific to EGM (-). The BRS GM-100 has a company reported accuracy range of 90%.

Sixteen aliquots of CM were removed from the -80°C freezer and allowed to warm at room temperature for two hours. Two readings were taken from each aliquot, and then placed into the concentration correction sheet generated by the company from the standard curve data. The corrected concentrations were then averaged. The EGM (-) readings were taken from the standard curve data, converted by the correction sheet, and reported below.

2.2.8. Statistical Analysis

Statistical analysis was conducted with R Statistical Software (R version 4.3.2 (2023-10-31)) (R Core Team, 2023). If a follicle demonstrated -5% to 5% static growth, it was normalized to 0% growth. If a follicle demonstrated negative growth for a timepoint without qualifying as dead, the follicle was included in that day's average. Growth averages per day were then analyzed via Kruskal-Wallis test with significance set at $p \leq 0.05$. Pairwise comparisons were performed with a Wilcox test and a Benjamini Hochberg p value adjustment.

Survival was analyzed with respect to treatment and initial size with a Cox's proportional hazards test, with the survival package in R, p value significance at $p \leq 0.05$ (Therneau & Grambsch, n.d.). It was then plotted via the survminer package (Kassambara et al., 2021). If significance between curves were found, the emmeans package was used to test pairwise comparisons (Lenth, 2023).

A chi-squared test was performed to determine any differences in oocyte maturation rates. Significance was set at $p \leq 0.05$.

A one sample t-test was performed to check for significant differences in glucose concentration, with a significance set at $p \leq 0.05$.

2.3. Results

2.3.1. Culture condition and follicular stage effects on follicle survival and growth

To identify ideal culture conditions for follicular culture, follicles were grown in co-culture or varying degrees of CM. Culture condition influenced follicular survival (Figure 2.1, $p < 0.05$). Specifically, follicles incubated in 100% CM exhibited a higher survival rate than the control, 20% CM, and 50% CM groups, while the co-culture treatment displayed intermediate

survival rate. At Day 13, the 100% CM group displayed a survival rate of 47% (15/32 follicles) compared to 33% (11/30) in the co-culture treatment, and 20-30% survival in other conditions ($p = 0.0024$). The contrast is even more pronounced by days 8 and 11. Specifically, 88% (28/32) and 78% (25/32) of follicles incubated in 100% CM survived on Day 8 and 11, respectively, but less than 60% did so on Day 11 in other groups.

Initial follicle size also impacted follicular survival (Figure 2.2, $p \leq 0.0001$). Antral follicles displayed the lowest survival rate at Days 8 and 13, although there were no differences between preantral and early antral follicles. The survival rates at Day 8 were 79% (66/83), 80% (44/55), and 59% (10/17) for preantral, early antral and antral follicles, respectively. By Day 13, only 12% (2/17) of antral follicles remained viable compared to 42% (23/55) for early antral and 31% (26/83) for preantral follicles.

Culture condition did not impact growth rates ($p > 0.05$) (Figures 2.3, 2.4). Initial size also did not have any significant impact on growth rates (Figure 2.5).

Visual assessments of the seeded co-culture cells showed increasing density of cells over time (Figure 2.6). As culture proceeded, cells began to aggregate, and the plasma membranes became irregular. Morphologically, the cells also shifted from a mixture of elongated, fibroblast-like and cobblestone-like cells, to only long, thin fibroblast-like cells. On average, cell confluency reached 80% and greater by Day 6 or Day 8 of culture. Once highly confluent, cell morphology changes and death increased. This coincided with lowered survival rates in the co-cultured follicles as compared to 100% CM follicles. Overall, 100% CM maintained higher survival rates in preantral and early antral feline follicles, best up to 11 days.

2.3.2. Impact of *in vitro* culture condition on gene expression

To identify if culture conditions impacted follicle development, expression of three key follicular health markers were measured. *In vitro* culture condition influenced expression of *CYP19A*, but not *FSHR* and *GDF9* (Figure 2.7). Specifically, *CYP19A* expression increased almost three-fold in the 50% CM treatment group over the control follicles ($p \leq 0.05$). However, there were no differences among other culture treatments in *CYP19A* expression. No treatment group showed a significant difference in expression of all three genes that would indicate better follicular health.

2.3.3 Impact of *in vitro* culture condition on oocyte maturation

To see if a meiotically mature oocyte could be obtained, follicles were cultured with ovarian stromal cells and CM for 11 days. Culture condition did not impact developmental competence of recovered oocytes. There were no oocytes that achieved metaphase II after *in vitro* culture and maturation. Further, there were no differences in rates of GV, GVBD, and degenerated oocytes (Figure 2.8, $p > 0.05$). Percentages of oocytes at each stage are shown in Table 2.3. Average initial diameter for follicles that survived to Day 10 (as defined above in Section 2.2.4) was 367.9 ± 10.1 μm, while their Day 10 average diameter was 409.4 ± 11.1 μm (mean ± SEM). Follicles at the stage investigated were likely not able to fully mature an oocyte.

Table 2.3: Oocyte Maturation Status of Oocytes Recovered From *In* Vitro Cultured Follicles. Percentage of germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase II (MII), or degenerated (D) oocytes recovered from control, co-culture and 100% CM follicles and subjected to *in vitro* maturation for 24 h.

Treatment	**Total Oocytes**	**GV (%)**	**GVBD (%)**	**MII (%)**	**D (%)**
Control	43	9.3	46.5	0	41.9
Coculture	46	6.5	47.8	0	39.1
100% CM	43	14	53.5	0	25.6

2.3.4. Glucose concentrations of CM and EGM (-)

To see if reduced glucose concentration in CM lowered metabolic stress in follicles and improved survival, glucose concentrations in CM were measured. There were no significant differences in glucose concentration between CM and EGM (-) ($p > 0.05$). Corrected readings of glucose concentration are presented in Table 2.4. Therefore, it is unlikely that glucose concentrations contributed to the 100% CM treatment group's increased survival.

Table 2.4: Glucose Concentrations of Conditioned Media. The average of corrected concentrations from EGM (-) and sixteen aliquots of CM that were allowed to equilibrate to room temperature for two hours.

Aliquot	Corrected Concentration Average (mmol/L)
EGM (-)	5.2
CM 1	4.35
CM 2	6.25
CM 3	3.15
CM 4	5.2
CM 5	6.2
CM 6	4.45
CM 7	6.4
CM 8	6.8
CM 9	6.7
CM 10	6.2
CM 11	5.6
CM 12	6.0
CM 13	5.9
CM 14	5.6
CM 15	5.1
CM 16	5.1

Figure 2.1: Percent Survival of Cultured Follicles by Treatment Group. Survival curve of follicles based on treatment groups 100% CM, 50% CM, 20% CM, co-culture, and control (n = 10 replicates, 155 total follicles). Cox proportional hazards test, p = 0.0024.

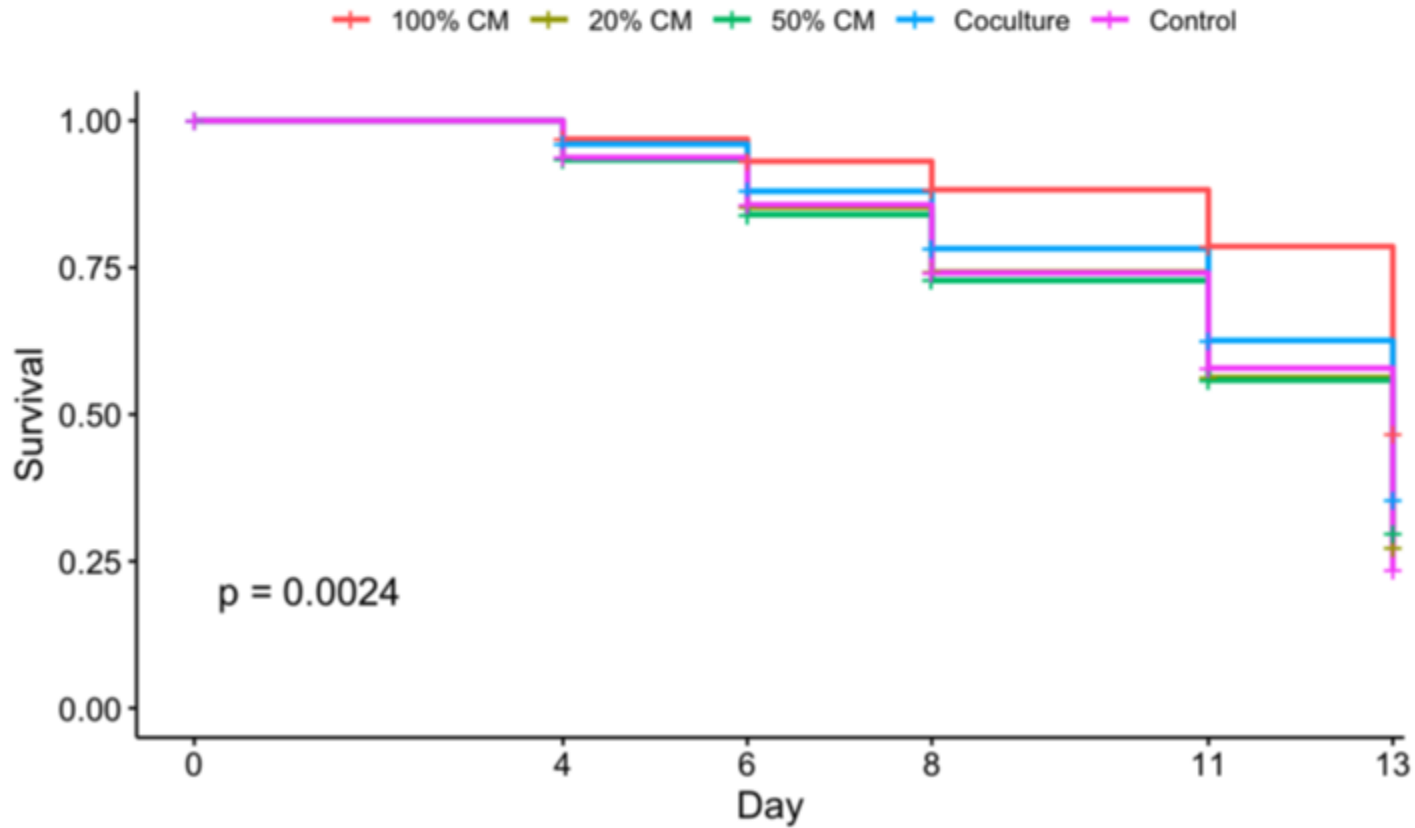


Figure 2.2: Percent Survival of Cultured Follicles by Initial Size Classification. Survival curve by follicle initial size categorization of preantral, early antral, and antral (n = 10 replicates, 155 total follicles). Cox proportional hazards test, $p < 0.0001$.

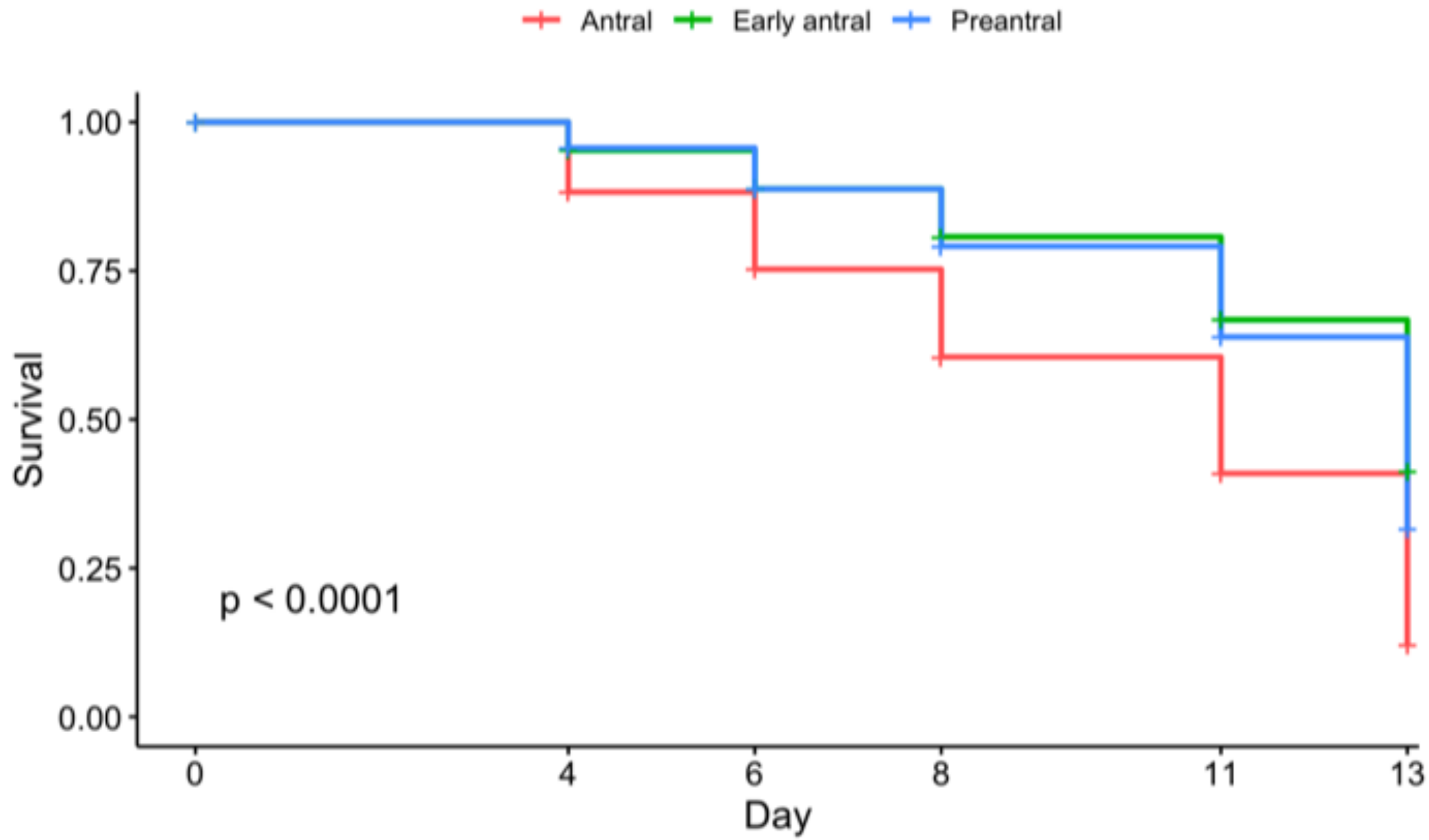


Figure 2.3: *In Vitro* Follicle Growth. A) Representative follicle's growth and antral expansion over the culture period. The oocyte is visible in the center of the follicle. Total follicle growth was 45.77%. B) Representative follicle death. Note the size reduction and blurred edges. The oocyte is also not visualizable. Scale bar is 500 μm.

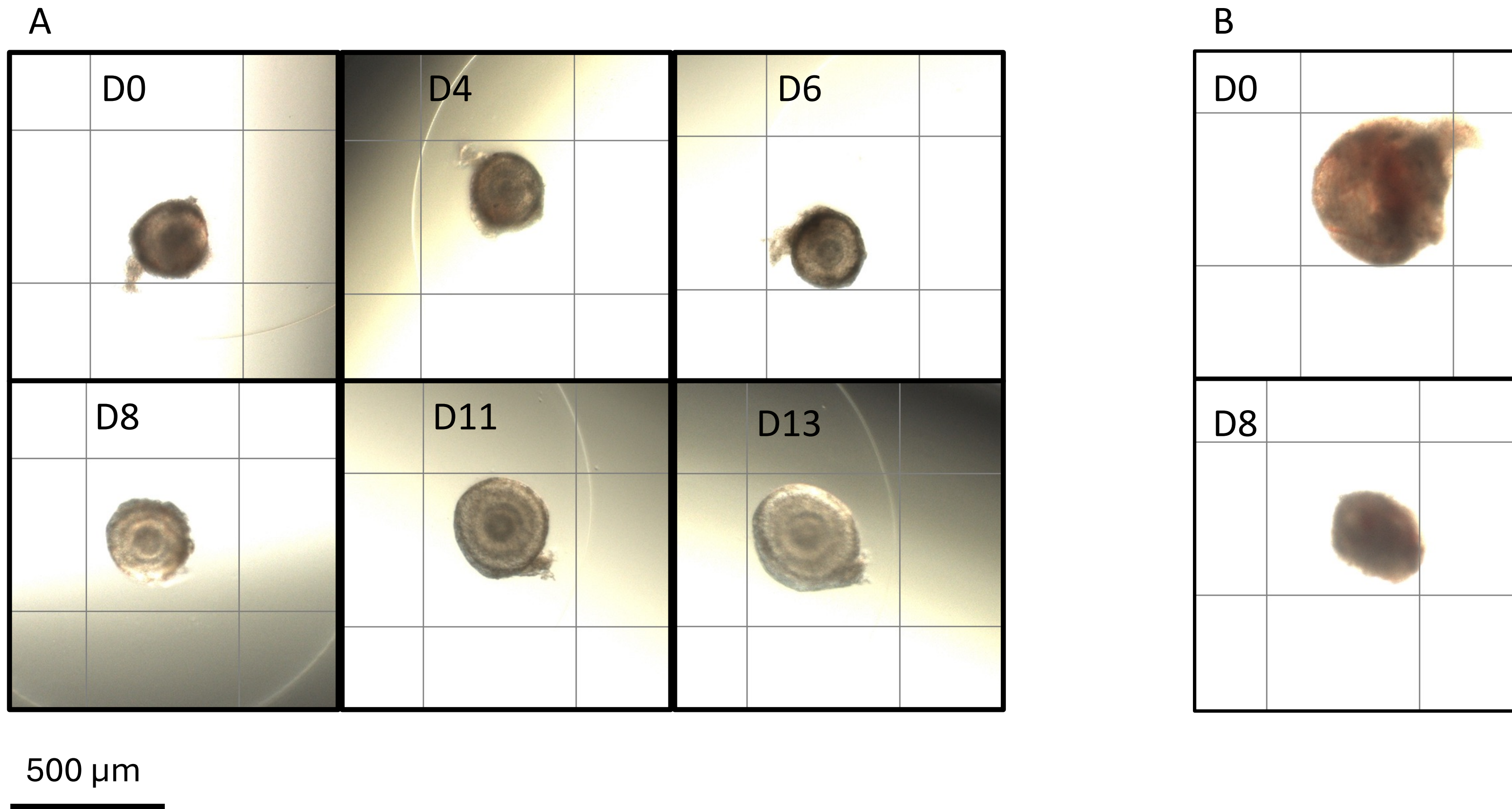


Figure 2.4: Percent Growth of Cultured Follicles by Treatment. (A – C) box and whisker plot of percent growth of cat follicles on day (D) 4, 8, and 13, divided by treatment group (n = 10 replicates and 155 total follicles, p > 0.05).

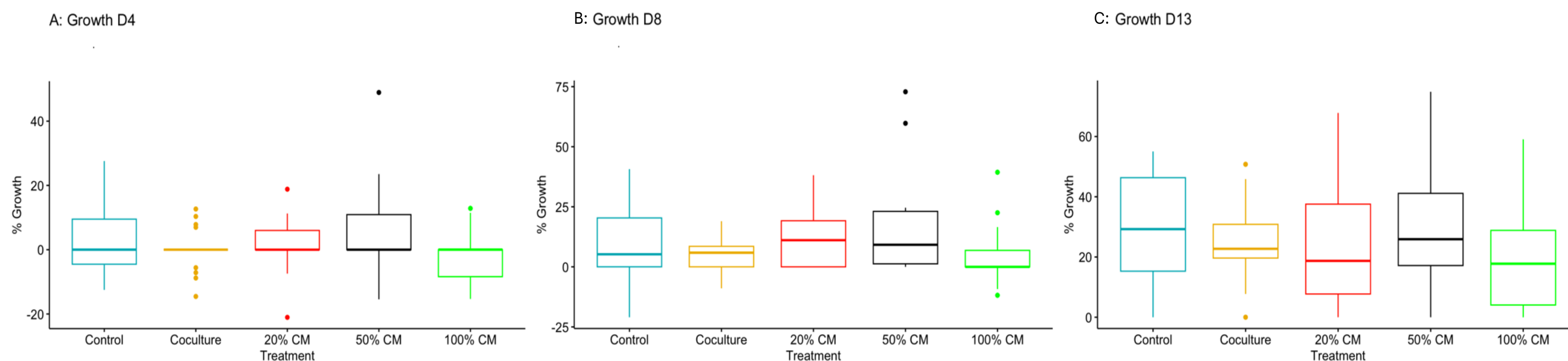


Figure 2.5: Percent Growth of Cultured Follicles by Initial Size Classification. (A- C) box and whisker plot of percent growth on day (D) 4, 8, and 13, divided by initial size categories (n = 10 replicates and 155 total follicles, p > 0.05).

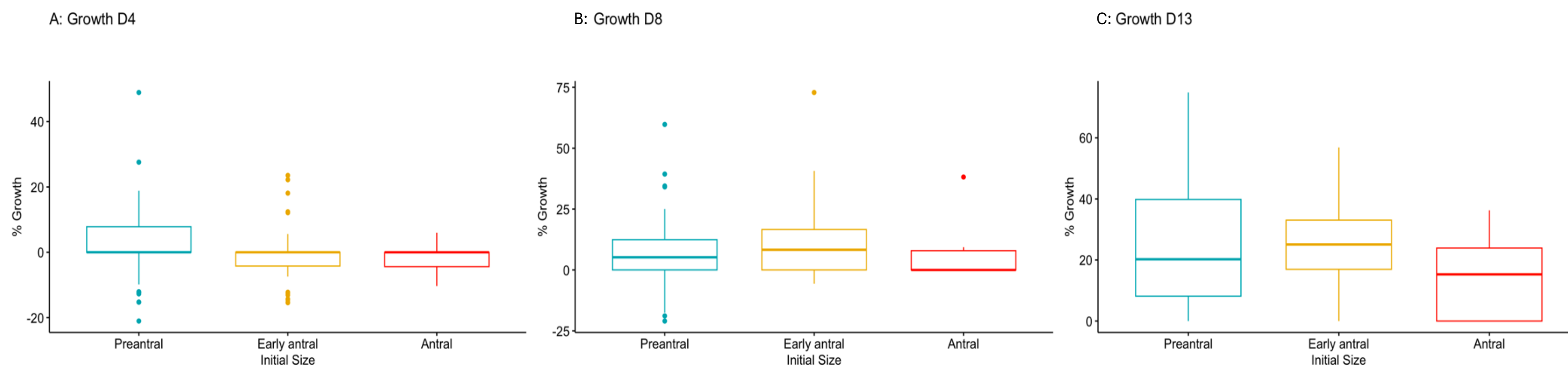


Figure 2.6: Co-Culture Cell Growth. (A – D) representative pictures of co-culture cell growth over the culture period. Scale bar is 500 µm. Note that on Day 0, the cells are sparsely populated and mostly fibroblastic in shape. By D6, cells have not quite reached full confluency, but cell membranes have become more irregular. On D8, cells have reached 80% or greater confluency. On D13, cells are overgrown, their morphology striated due to crowding.

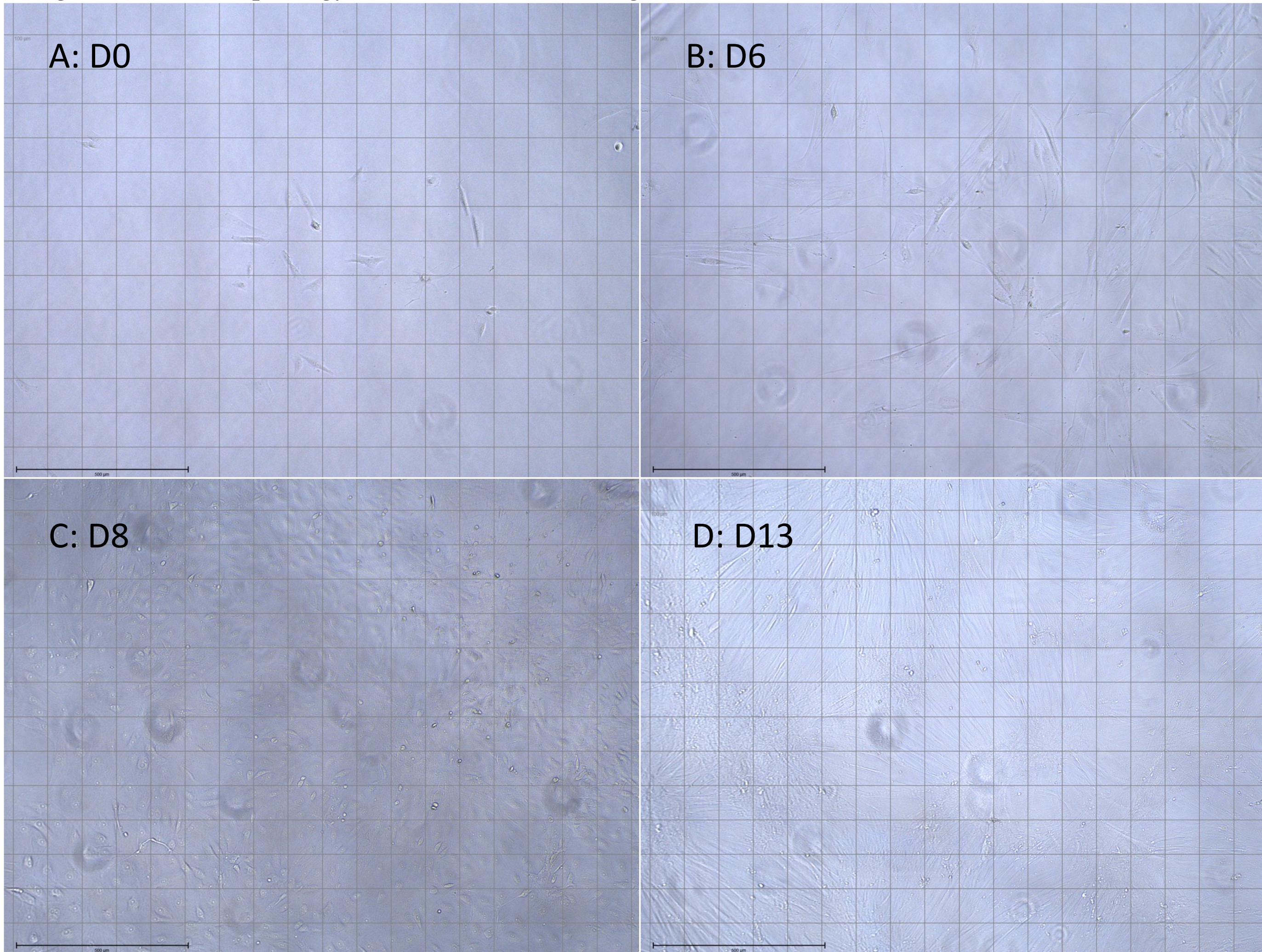


Figure 2.7: qRT-PCR Analysis of *In Vitro* Cultured Follicles. Gene expression of *CYP19A*, *FHSR* and *GDF9* of freshly isolated or 13 days *in vitro* cultured follicles (n=3 replicates). Expression is relative to cultured control and normalized to *β-actin*, $p \leq 0.05$ (*).

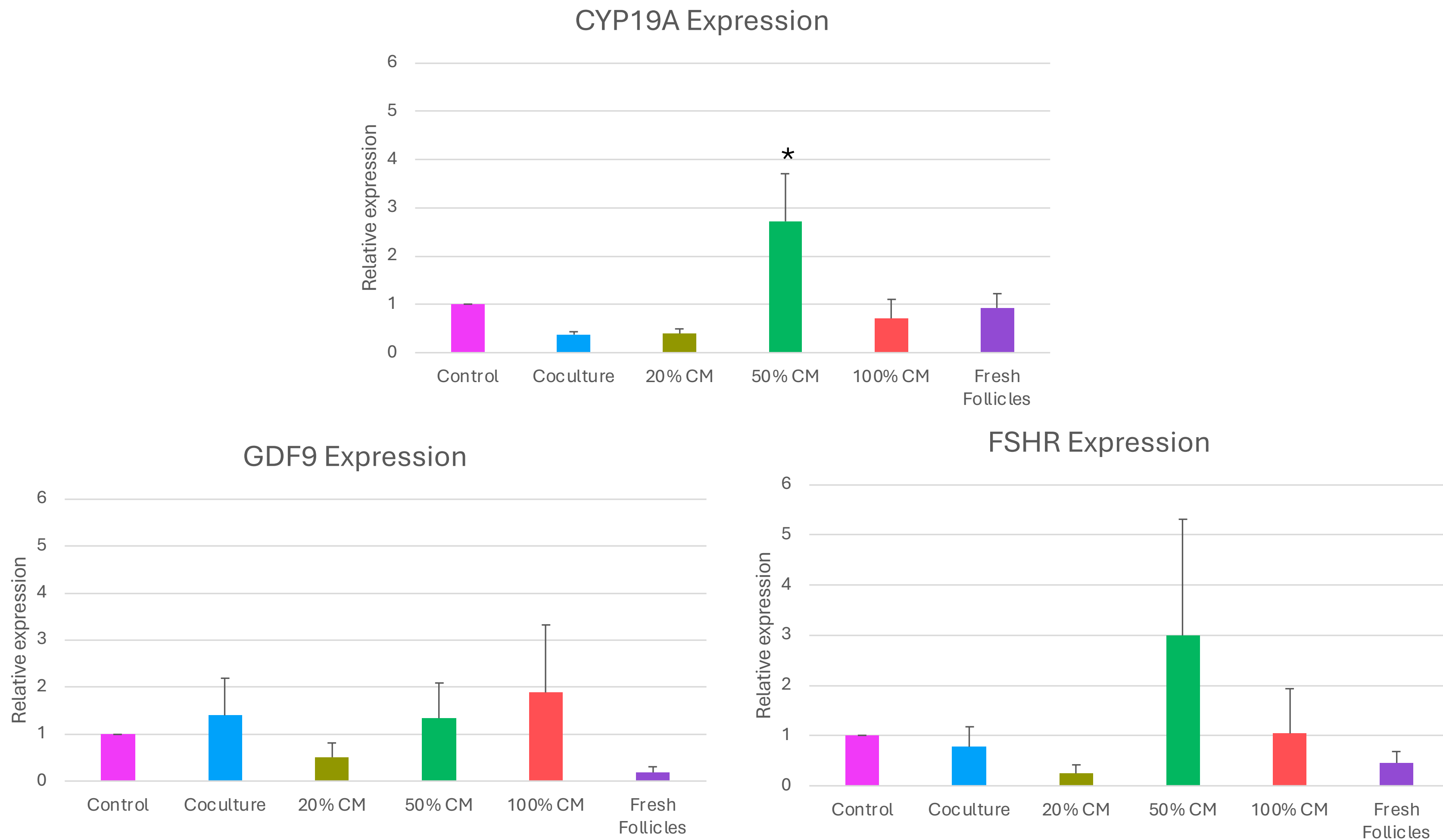
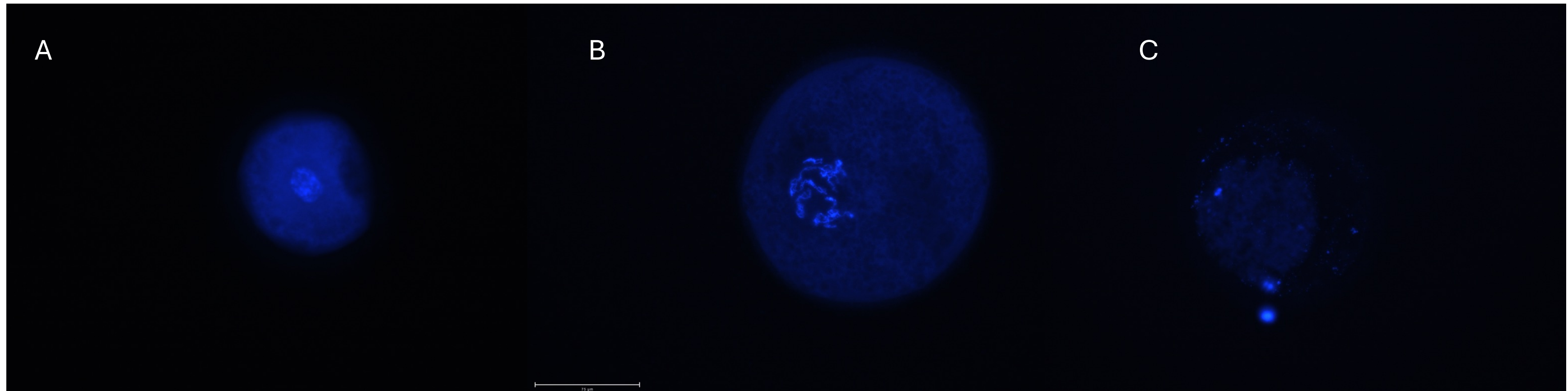


Figure 2.8: Representative Pictures of *In Vitro* Matured Oocytes. Oocytes fixed and stained with DAPI at different maturation points (n=11 replicates, 199 total follicles, 132 total oocytes). Scale bar is 75 µm. A: Germinal vesicle. B: Germinal vesicle breakdown. C: Degenerated.



2.4. Discussion

In vitro culture conditions have been shown to influence the survival and development of isolated ovarian follicles in many species (Simon et al., 2020). In the present study, I investigated the impact of ovarian stromal cells on the growth and survival of isolated preantral, early antral and antral cat follicles. I found that 1) 100% conditioned medium from ovarian stromal cells best supported follicular survival in a 13-day culture period; 2) initial developmental stage of follicles affected survival outcomes; 3) ovarian stromal cell and conditioned medium supplementation did not improve follicle growth nor oocyte maturation rates; and 4) *CYP19A* expression was upregulated in 50% CM treatment.

Previous studies have shown that co-culturing oocytes and embryos with follicle cells, oviductal cells, or feeder cells improve gamete and embryonic development (Desai et al., 2007; C. I. Kim et al., 1990; Virant-Klun et al., 2018). Pig cumulus-oocyte complexes (COCs) that were cultured with either human endothelial progenitor cells (EPC) or CM from the EPC population had higher rates of oocyte maturation and embryonic development than COCs that were not cultured in these conditions (Lee, 2020). Recently, one study has shown that ovarian stromal feeder cell co-culture improved both the survival and the growth of human preantral follicles (Grubliauskaitė et al., 2024).

Ovarian stroma cells produce factors that regulate folliculogenesis, including VEGF, FGF2, IGF-1, and EGF (Lee, 2020). These factors likely contributed to improved follicle survival observed in the present study, in which 100% CM improved survival significantly. However, there were no significant differences in survival and growth between co-cultured follicles and the control (Figures 2.1, 2.4). Nonoptimal cell culture techniques may have negated some beneficial

effects of co-culture. It is known that extended intervals between passages affects cell growth, differentiation, and necrosis rates, even as high passage numbers can alter secretome expression and affect conditioned media composition (Park et al., 2022; Topman et al., 2011). In Grubliauskaitė *et al.*, follicle culture lasted for seven days after seeding cells at a fixed number (Grubliauskaitė et al., 2024). Here, cells were also seeded at a fixed number, but then allowed to grow for 14 days (one day to adhere and 13 days of follicle culture). As the culture progressed, the cells visibly and consistently changed morphology, becoming irregular and elongated, with cell aggregation as they competed for space. Based on visual assessments, the morphology changes and confluency occurred on average by Day 8 (Figure 2.6). This is consistent with Grubliauskaitė *et al.*'s assessment of confluency, and further corresponds to a drop in co-culture follicle survival as compared to 100% CM. It is likely that these stressed cells were secreting necrotic factors, such as death ligands that are members of the tumor necrosis factor family, or concentrations of dissolved gases, such as nitric oxide, into the follicular culture (Elmore, 2007). Nitric oxide has been previously shown to negatively affect nearby cells in an overgrown co-culture schematic (Groot et al., 2003). With increasing necrotic factors in the follicle culture, follicles likely could not survive, despite potential growth factors added by healthy cells. Future studies to assess nitric oxide production, necrotic factors, and reactive oxygen species will give valuable insights in how to improve the coculture system used here.

After observing the beneficial effect of 100% CM on preantral and early antral follicle survival, I hypothesized that nutrient modulation in CM versus EGM (-), including glucose concentration, could contribute to the observed survival rates. Glucose and pyruvate are the main metabolites for ovarian follicles and oocytes, respectively. In antral follicles, glucose is metabolized primarily through glycolysis, which reduces oxygen demands for the rapidly

expanding follicle (Harris et al., 2007). Findings obtained from previous studies suggest that excess nutrients, especially carbohydrates, could cause metabolic stress in follicles, oocytes, and embryos, that in turn compromise development (Harris et al., 2007; Herrick et al., 2020; Krisher et al., 2015). For example, a 50% reduction of carbohydrates results in normal development of mouse embryos at a comparable rate to the control, while cleaved bovine embryos were more likely to reach blastocyst stage (Ermisch et al., 2020; Santos et al., 2021). Because cultured cells consume glucose at a high rate, perhaps CM had a lower glucose concentration than the EGM (-) used in other cultured groups (Torimoto et al., 2022). However, tested CM glucose concentrations averaged 5.5 ± 0.24 mmol/L, and there was no significant difference between CM and EGM (-). Therefore, it is unlikely that lowered glucose concentration played a role in 100% CM follicle survival.

Growth is unaffected by treatment in this experiment. However, this result is not universal. While there are other studies where follicle growth is unaffected by CM or co-culture, others show that coculture or CM can affect growth (Choi et al., 2013; Kedem et al., 2017). It is important to note that many studies have vastly different setups, such as different cell types, different culture periods, and different follicle stages. All these factors can impact follicle development outcomes. One aspect likely limiting growth in this study, though, is the alginate beads. While the alginate does provide needed structure to the follicle, it also restricts growth in antral follicles(Vanacker & Amorim, 2017). Alginate does not naturally biodegrade, and therefore the follicle cannot remodel the alginate as it would the cortex *in vivo*. To allow antral expansion, fibrin has been added to alginate beads in other studies, as a biodegradable material that can be remodeled by follicular enzymes, like the ovarian cortex *in vivo* (Shikanov et al., 2009). Further, although developmental stage did not impact follicle growth, it should be noted

that there were large discrepancies in the numbers of follicles in each developmental stage (preantral, 83; early antral, 55; and antral, 17) which likely skewed analysis.

Oocyte maturation did not reveal any significant differences between treatment groups, nor did it yield a metaphase II oocyte (Figure 2.8). Historically, rates to obtain a metaphase II feline oocyte have been approximately 50% of *in vivo* grown and *in vitro* matured oocytes (Johnston et al., 1989). It is important to note that, in this study, oocytes were collected from follicles regardless of survival status, as long as an oocyte was present within the follicle. This may have increased the relative numbers of degenerated oocytes from follicles that had constricted growth. Also, follicles that survived to Day 10 were, on average, 409 μm in diameter. Previous studies have shown that the percentage of morphologically normal oocytes that can be recovered from 400 – 800 μm feline follicles is around 20%, depending on estrous cycle stage (Uchikura et al., 2011). After *in vitro* maturation of those morphologically normal oocytes, 28% to 38% were degenerated, 37% to 54% were classified as GV, and 10% to 23% reached metaphase II (Uchikura et al., 2011). Therefore, that no oocyte reached metaphase II in this study is unsurprising. The follicles were likely not mature enough to produce a developmentally competent oocyte. Further, the culture period may have contributed to lowering mature oocyte numbers. Obtaining a follicle that can produce a developmentally competent oocyte is therefore a delicate balance between culture length, initial follicle size, and potential growth. If a follicle is too large initially, then it will likely die, as evidenced by antral follicle survival rates in this study (Figure 2.2). Yet a follicle that is too small may not grow enough in the culture time period, and chances of survival decrease the longer the culture continues. Solving this dilemma may include designing a multistep culture system or decreasing the alginate concentration to allow follicle expansion as *in vitro* culture progresses.

qRT-PCR analysis showed an upregulation in *CYP19A* expression in 50% CM follicles. CYP19A, or aromatase, is the final enzymatic step for production of estrogen(Chen et al., 1988). This finding was unexpected, as 50% CM improved neither follicle survival nor growth. Variations between follicle sizes in each qRT-PCR replicate may contribute, although it is unlikely to be the complete explanation of why *CYP19A* was upregulated. Further, upregulation of mRNA expression does not necessarily correlate to increased translation and protein production. *In vitro* produced goat embryos showed an upregulation in *OCT4* mRNA expression, but a decreased OCT4 protein expression (He et al., 2004). Perhaps 50% CM culture induced a similar phenomenon in the follicles, leading to higher mRNA expressions without producing a downstream effect on growth or survival. More research is required to elucidate these results.

In conclusion, these results indicate that ovarian stroma cell conditioned media has a positive impact on survival in feline pre- and early antral follicles. This finding serves as a foundation for future studies aimed at exploring survival factors secreted by ovarian stromal cells, vital information for developing an ideal *in vitro* microenvironment for feline folliculogenesis.

Chapter 3 – Future Directions and Concluding Remarks

This study showed the positive impacts of conditioned medium on improving survival, but not growth, of domestic cat pre- and early antral follicles. This is the first study, to my knowledge, that explores the benefit of ovarian stromal cells as a co-culture and conditioned medium on *in vitro* development of cat follicles. While the present study demonstrated potential benefit of conditioned medium, there remain several avenues that warrant further investigation, including co-culture improvements and conditioned medium secretome analysis.

In the present study, I observed that the co-culture cells became consistently overgrown as *in vitro* follicle culture proceeded. This may have had negative effects on the follicles, as evidenced by their lower survival compared to the 100% CM treatment. One alternative approach is to seed new wells with ovarian stromal cells, then move the follicles into them every few days. However, this approach also has a risk of over handling follicles, which can result in mechanical damage and compromised development. Blocking mitosis of the seeded cells, via either chemicals like mitomycin C or via gamma irradiation to prevent cell overgrowth is another potential alternative(Llames et al., 2015). However, there also is a risk of altering cell function that in turn compromising their ability to support follicle development. Therefore, further characterization of blocked ovarian stromal cells is warranted.

Although paracrine factors were hypothesized to be secreted into the CM, there is no direct evidence for it in this study. Growth factors found in CM from other studies include VEGF, FGF2, IGF-1, and EGF, and these were theorized to directly contribute to an increase in oocyte maturation and embryonic development(Lee, 2020). Other factors that might have influenced survival may include exosomes and antioxidants(Huang et al., 2018; Ra et al., 2023). However, the method chosen for detection of proteins in the CM, Western blot, did not conclusively reveal

the presence or absence of any growth factors (data not shown). One issue that arose was antibody cross reactivity. Since the growth factors were produced by cat cells, antibodies raised against mouse or human proteins did not always bind appropriately. Of the five factors tested for (FGF2, FGF7, BMP4, VEGF, and TGFβ), only three antibodies bound a general ovarian tissue lysate (FGF2, BMP4, and VEGF). When tested against CM, no signals were detected. One possible reason for this is that the targeted proteins were present at a very low concentration that in turn cannot be detected by the Western blot analysis used. Optimization of the protocol for sample preparation and antibody affinity, along with more sensitive Western blot methods is required to fully explore what paracrine factors are present, including growth factors, antioxidants, and ROS. Alternatively, other methods may be considered, such as mass spectrometry.

Lowered nutrient concentrations have been shown to reduce metabolic stress and improve maturation and survival in follicles, oocytes, and embryos (Herrick et al., 2020; Krisher et al., 2015). This may be another factor to 100% CM's beneficial effects. However, since the glucose concentration tests were performed as a post study investigation to explain why the 100% CM treatment group follicles survived the best, the sampled CM may not provide the most accurate picture. A future study should further explore glucose concentration effects on follicles, by comparing treating follicles with 100% CM and a high glucose concentration versus treating follicles with 100% CM and a low glucose concentration. Measuring the available glucose in CM before follicle culture, then measuring the discarded media at each feeding would further provide context for how much glucose feline follicles require. Another avenue to investigate the overall effects of lowered nutrients on reducing follicle metabolic stress, intertwined with protein analysis for growth factors present in CM, is to characterize protein composition of CM. During

Western blot analysis, EGM (-) was found to have 26 μg/μL of protein, while CM had less than 1 μg/μL of protein (data not shown). Future investigations should focus not only on growth factors in CM, but also availability of free amino acids to see how much nutrients is available to follicles.

Assisted reproductive technologies, such as *in vitro* folliculogenesis, are crucial for fertility preservation and preventing genetic bottlenecks in captive bred wild species. By applying the results of this study and further exploring the benefits of conditioned medium, progression of feline conservation efforts is possible.

Appendices

Pilot Study – Introduction and Materials and Methods

Previously, a MEM based culture medium has been used to culture isolated feline follicles (Fujihara et al., 2014). However, it was unknown if the ovarian stromal cell population would survive in MEM, given previous lab protocol for culturing the cells in EGM. Therefore, a pilot study and transition phase were conducted, with two main objectives: 1) to investigate the effects of transitioning ovarian stromal cells from EGM to MEM, specifically related to the cells' ability to proliferate and maintain population characteristics; and 2) to investigate follicle survival and growth in both EGM and MEM.

Ovarian stromal cells were recovered from cryopreservation as described in Chapter 2.1.2, cultured in EGM, and passaged when the cells achieved 80% confluency. After the first passage, the cells were divided into two groups: control and transition. Cells in the control group were maintained in EGM with medium replaced every 48 hours and passaged when achieving 80% confluent. In the transition group, culture medium was removed every 48 hours and replaced with a mixture of EGM and MEM (alpha MEM supplemented with ascorbic acid 1 μg/mL, penicillin 50U/mL, streptomycin 0.05 mg/mL, and 2% FBS). EGM was subsequently decreased at each time point by 25%, until the medium was entirely comprised of MEM. After full MEM transition, cells were maintained until the second passage.

Follicles (n = 62, 8 cats) were encapsulated, divided into two treatment groups – MEM (n = 30) and EGM (n = 32) – and cultured for 11 days. Pictures were taken on days 0, 4, 6, 8, and 11 of culture. Follicles were also fed on these days, by removing half of the medium and replacing it with fresh.

Growth and survival status were defined as in Chapter 2.1.4. If a follicle demonstrated two consecutive assessments of negative growth by day 6, the follicle was discontinued from culture.

Pilot Study – Results and Discussion

Over the course of four replicates in four different cell lines, none successfully made the transition from MEM to EGM (Table A.1). Two lines of cells had been cryopreserved, while two were freshly isolated. Based on visual assessment (Figure A.1), the two fresh cell lines died at the 50% transition mark. However, one cryopreserved cell lines survived the transition entirely, only to die after the first passage. The other line did not survive once fully transitioned to MEM. Meanwhile, all control lines survived until the end of incubation.

Follicular growth was not significantly different between treatment groups, except on day 11 ($p = 0.05$). Follicles cultured in MEM exhibited a higher growth rate than those in EGM on day 11, with a mean growth percentage ± standard deviation of 50.67% ± 35.6 and 20.36% ± 14.3, respectively (Figure A.2). Initial size also did not have a significant impact on growth rate (Figure A.3). Finally, follicle survival was not impacted by culture medium ($p > 0.05$) (Figure A.4).

Because the ovarian stromal cells could not survive in MEM and there were no differences in follicle survival between the two media, EGM was selected as the base medium to investigate ovarian stromal co-culture effects on follicle development.

Table A.1: Ovarian Stromal Cell Survival During Media Transition. Survival time points for transition from EGM to MEM. Green check marks denote cell survival while red X's denote cell death at that time point.

Replicate	Source	100% EGM 0% MEM	75% EGM 25% MEM	50% EGM 50% MEM	25% EGM 75% MEM	0% EGM 100% MEM	1st passage after 100% MEM
1	Frozen	✅	✅	✅	✅	✅	❌
2	Fresh	✅	✅	❌			
3	Fresh	✅	✅	❌			
4	Frozen	✅	✅	✅	✅	❌	

Figure A.1: Ovarian Stromal Cell Viability During Media Transition. (left) Replicate 4, day 3 after 100% MEM transition. Note the non-viable cells (arrows) and low cell density. (right) Replicate 4, day 3 after passage 5, EGM control.

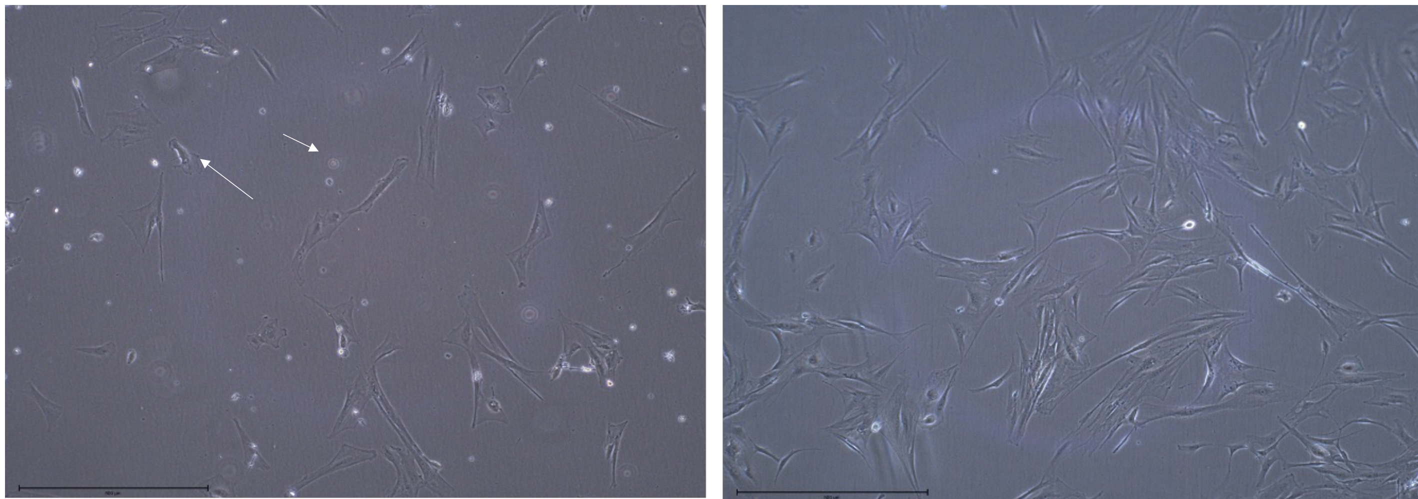
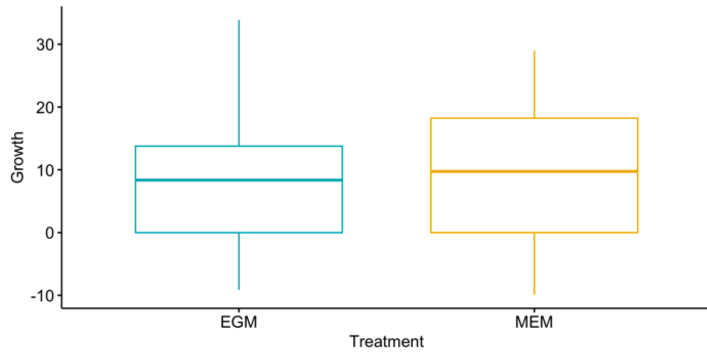
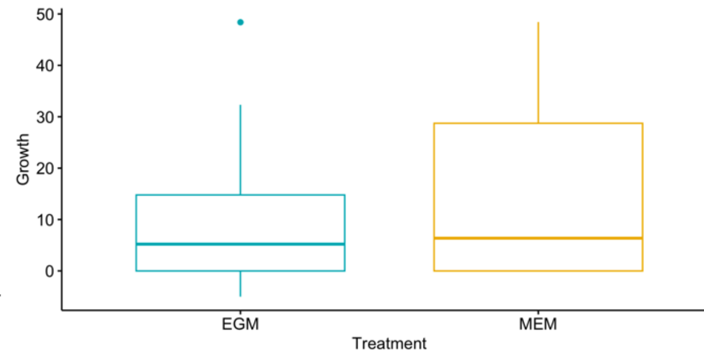
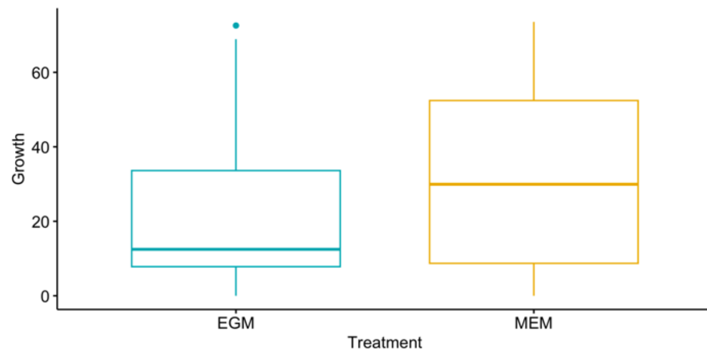


Figure A.2: Percent Growth of Pilot Study Follicles by Culture Medium. Box plot of percent growth of follicles on days (D) 4 (A), 6 (B), 8 (C) and 11 (D) in EGM and MEM based media. (*) indicates significant difference between the two treatments ($p < 0.05$).







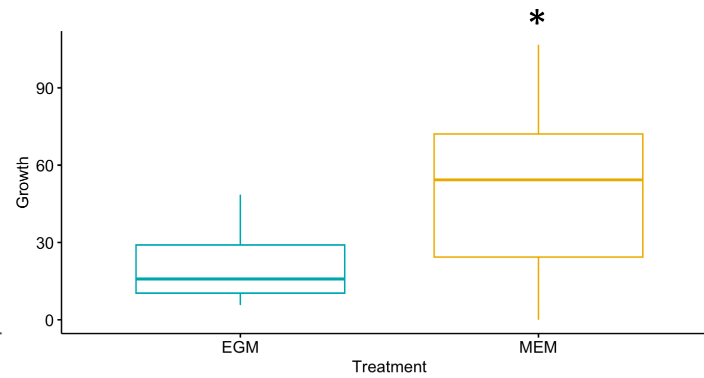
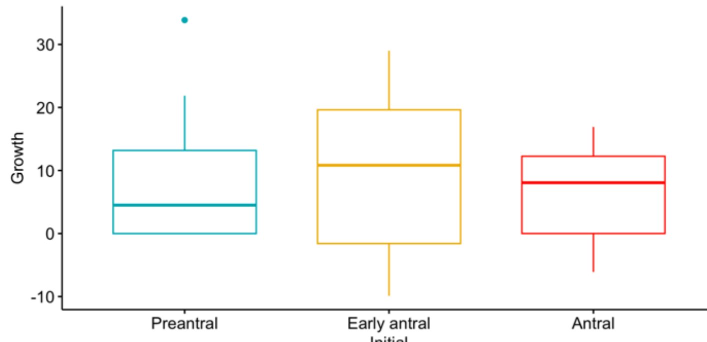
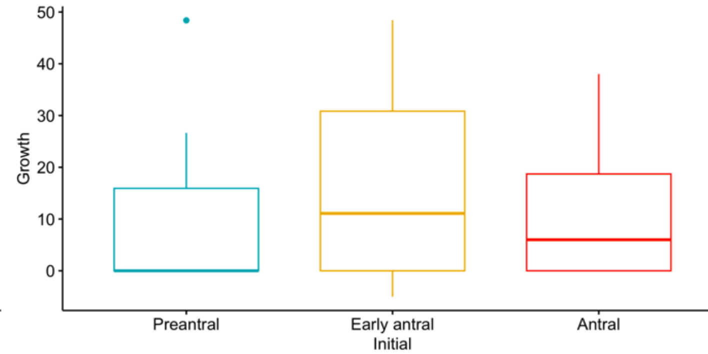
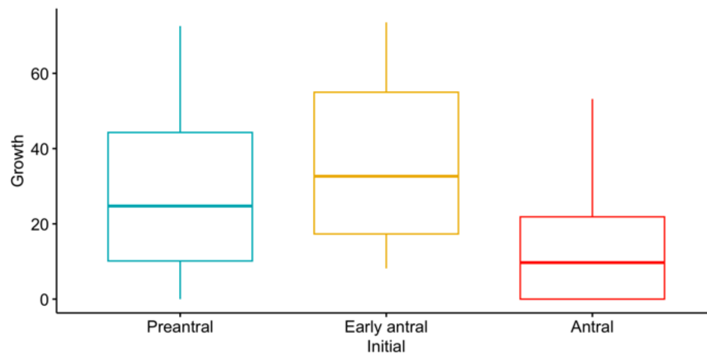


Figure A.3: Percent Growth of Pilot Study Follicles by Initial Size. Box plot of percent growth of follicles on days (D) 4 (A), 6 (B), 8 (C) and 11 (D) divided by initial average diameter classification.







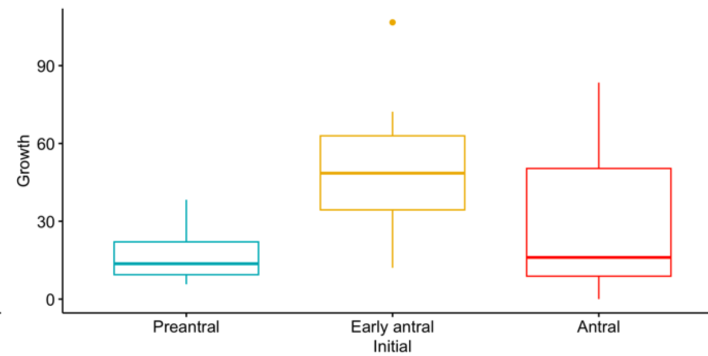
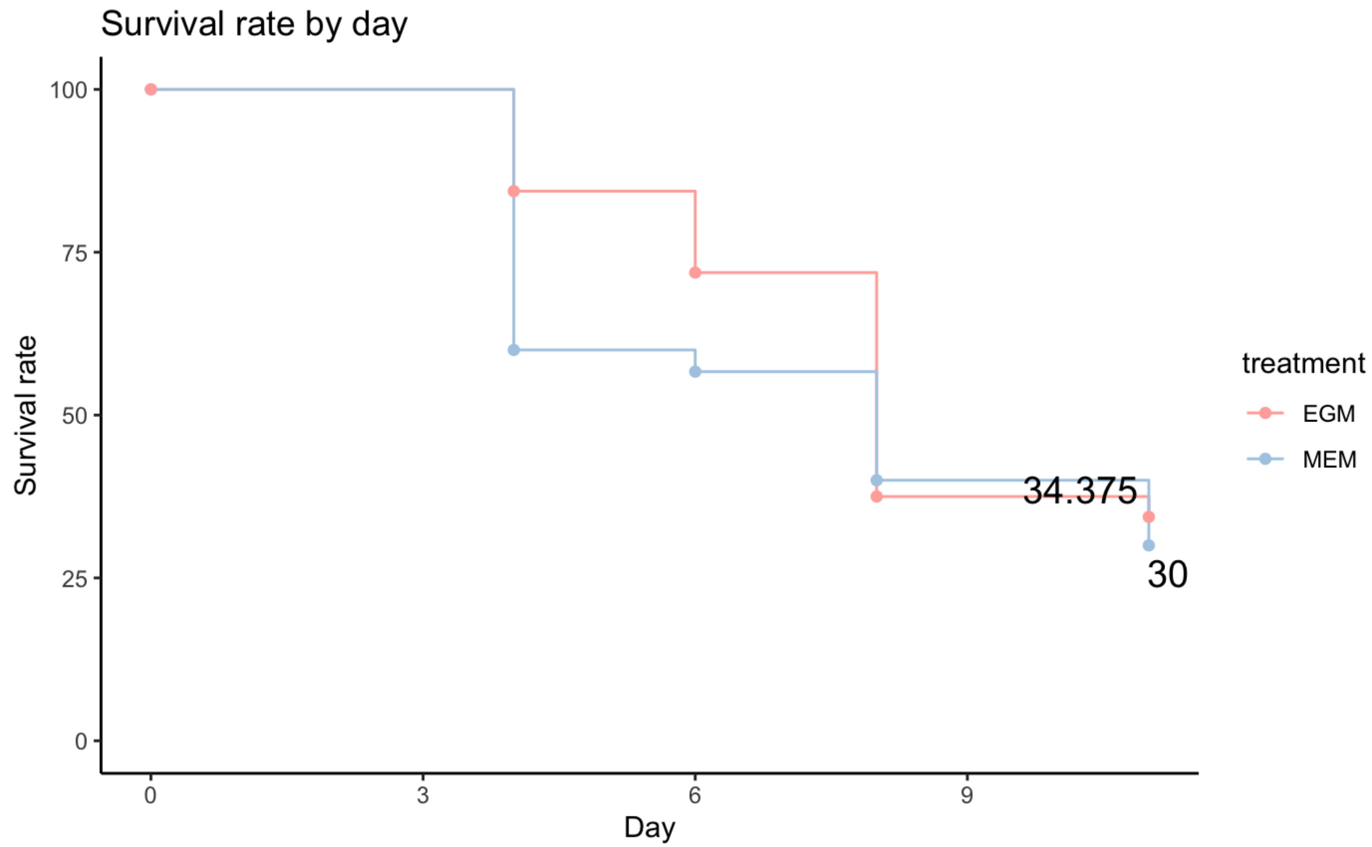


Figure A.4: Percent Survival of Feline Follicles by Culture Medium. Cat follicle survival rate based on culture medium, $p > 0.05$. Final survival on D11 was 34.375% for EGM and 30% for MEM.



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