FOLLICULOGENESIS AND FERTILIZATION IN THE DOMESTIC DOG: APPLICATION TO BIOMEDICAL RESEARCH, MEDICINE, AND CONSERVATION

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FOLLICULOGENESIS AND FERTILIZATION IN THE DOMESTIC DOG: APPLICATIONS TO BIOMEDICAL RESEARCH, MEDICINE, AND CONSERVATION

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Understanding of reproductive biology in canids, including the domestic dog, is surprisingly limited. This includes the regulators of ovarian follicle development, and mechanisms of anestrus termination, fertilization and embryo development. In turn, this lack of understanding has limited our ability to develop assisted reproductive technologies (ART) for endangered canid conservation efforts. ART of interest include in vitro follicle culture for maternal genome rescue, estrus induction protocols, and *in vitro* fertilization (IVF). Here, we describe: 1) Studies evaluating the stage-specific requirements for follicle stimulating hormone (FSH), luteinizing hormone (LH), and activin on domestic dog follicle development *in vitro*. We demonstrate the beneficial effects of FSH and activin on growth, and activin on antrum expansion and oocyte health in short term culture. 2) Evaluation of serum collected during the anestrus to estrus transition revealing a significant increase in anti-Müllerian hormone (AMH) during proestrus, likely originating from increased numbers of antral follicles during this time. 3) The birth of the first live puppies from IVF embryos utilizing *in vivo* matured oocytes. Further, consistently high rates of embryo production are obtained using the described system, with no effect of progesterone supplementation to embryo culture media. Improved knowledge of the drivers of follicle development inform understanding of the resumption of estrus cyclicity, which in turn is needed to explore mechanisms of oocyte maturation prior to fertilization. *In vitro* fertilization is also required to assess the developmental capacity of oocytes grown from cultured follicles. Thus, these combined discoveries represent significant advancements in the understanding of canid reproductive biology and potential application to development of ART for the management of biomedical research colonies and for endangered canid conservation.

BIOGRAPHICAL SKETCH

Jennifer Nagashima was born in Chigasaki, Japan but grew up in southern California. As a middle school student she became interested in endangered animal conservation, which she pursued through volunteer education and animal enrichment work at her local zoo. Following completion of an International Baccalaureate Diploma programme in high school, she attended Cornell University, receiving her B.S. in animal science with a minor in education in 2009. She then completed two internships with the Smithsonian Conservation Biology Institute (SCBI), researching African lion reproductive endocrinology and domestic dog ovarian follicle culture. Her experiences at SCBI introduced her to the world of reproductive research for animal conservation, which became a passion she pursued as the inaugural student of the Cornell-Smithsonian Joint Graduate Training Program (JGTP). Under the dual guidance of Dr. Alexander Travis at the Baker Institute for Animal Health at Cornell University and Dr. Nucharin Songsasen at SCBI, she began a Ph.D. program in the field of Zoology and Wildlife Conservation studying reproduction in domestic dogs for application to endangered canid conservation efforts. Upon completion of her Ph.D., Jennifer Nagashima will continue her research career as a Smithsonian Institution Postdoctoral Fellow in Front Royal, VA.

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CHAPTER ONE

Introduction and Literature Review

Introduction

Understanding of the regulation of several key aspects of reproduction, including estrous cyclicity, folliculogenesis, and fertilization/embryo development, are lacking in the domestic dog. This, in turn, has hindered our ability to control the reproduction of both domestic and wild canids, from the development of contraceptives to the advancement of assisted reproductive technologies (ART) to improve fecundity. This chapter will begin with the evaluation of the domestic dog as a research model. It will then review 1) our current understanding of reproductive cyclicity and status of intervention protocols in canids, 2) the current state of knowledge of ovarian folliculogenesis *in vivo* and *in vitro,* both in general and specific to the dog, and 3) mechanisms of fertilization/embryo development and progress made thus far in the domestic dog.

The domestic dog as a research model

1. For wild canids

The International Union for Conservation of Nature has designated 5/35 extant wild canid species as either endangered or critically endangered [1]. This number is likely to increase with the expanding threats of habitat loss, persecution, and the spread of diseases from domestic dogs (e.g. rabies [2, 3] and distemper [4]). As result, there is growing need for the creation of *ex situ* insurance populations. However, one critical challenge in the management of *ex situ* populations, is the maintenance of genetic diversity within a small group of animals over many generations. In pursuit of this goal, development of assisted reproductive technologies (ART) using the domestic dog as a model is a priority.

This lack of understanding of canid reproduction presents a problem for conservation efforts in endangered canids for two main reasons. First, should a female animal die during the non-breeding season (nearly 10 months of the year) there is currently no method for rescuing her genetics so they may be represented in the population. ART such as *in vitro* follicle culture

would be invaluable for this purpose. Immature follicles could be collected from the ovary even during the period of ovarian inactivity (i.e., outside breeding season and in prepubertal animals), and grown in stimulating conditions *in vitro* for the production of healthy, fertilizable oocytes for use in *in vitro* fertilization (IVF).

Second, for many canid species, there is only one short window of opportunity for natural breeding a year. In *ex situ* environments, which generally have only small populations of animals and attempt to breed specific individuals based on Species Survival Plan recommendations to maintain genetic diversity, this makes it extremely challenging to produce pups predictably in a timely manner. Natural breeding also requires, understandably, the animals to be at the same location, which means that achieving specific matches involves the often-stressful and expensive transport of valuable animals to distant locations. ART that would allow us to consistently control reproductive cycles in canids, either to improve our ability to time ovulation or to induce it, would greatly aid conservation efforts. Further, having a successful IVF system in place introduces the ability to transfer gametes or embryos to distant locations (often required to maintain genetic diversity in an *ex situ* breeding situation), rather than the animals themselves, and still produce live births. As the closest domestic relative, the dog is the ideal model to begin to understand its wild and endangered cousins for development of these technologies.

2. For biomedical research

Though most research in the field of human health uses the traditional laboratory mouse, there is increasing attention to the development of more relevant animal models for study [5, 6]. Human diseases are artificially induced in the mouse through knockout technology, unlike in the dog where many genetic afflictions occur naturally [7]. The organ and body size of the dog is more comparable to the human than the mouse model, making it better suited for this research. Domestic dogs also intimately share our environment, and thus are exposed to the same environmental factors that may contribute to disease. Further, with the complete sequencing of

the dog genome, the potential of this species as a biomedical model only grows [8]. However, to realize the dog's potential as a biomedical research model, the ability to produce specific transgenic animals would be required. Progress has been made in this area by performing somatic cell nuclear transfer with transduced fibroblasts expressing red fluorescent protein (RFP) [9] and doxycycline-inducible enhanced green fluorescent protein (eGFP) [10] as "proof of concept" experiments. However, these studies resulted in few transgene-carrying offspring, so there is a need to develop higher yield, lower cost methods for generating transgenic dog models.

3. For the domestic dog

Beyond the endangered canid conservation and biomedical research model applications of the domestic dog model, there is growing interest in understanding domestic dog reproduction in itself. There are two primary reasons for this. First, groups interested in both the preservation of rare dog breeds and the production of specific working dogs are invested at improving domestic dog reproduction. For example, the ability to yield more dogs per year that are adept at alerting for seizures is a high priority, as it contributes to the survival as well as the quality of life patients suffering from frequent seizures [11-13]. Conversely, the Michelson Prize highlights the serious need for the development of safe, inexpensive nonsurgical methods for domestic animal sterilization. Improved understanding of domestic dog reproduction is needed to inform these efforts. The availability of a low-cost, efficient contraceptive would not only help stem the growing population of stray animals but, in turn, reduce the spread of disease within this population and between domestic animals and wild populations. These contraceptives could also be utilized for the control of wild canid populations (e.g. foxes, coyotes [14]).

Reproductive Cyclicity

1. Overview

For most mammals, the estrous cycle can be broken down into four to five stages. Proestrus and estrus together make up the follicular phase, the key developmental period within which an ovarian follicle containing an oocyte (See Folliculogenesis section), will grow and become a dominant follicle. Follicle growth is dependent on follicle stimulating hormone, or FSH, produced by the pituitary in response to gonadotropin-releasing hormone (GnRH) pulses from the hypothalamus (Fig. 1.1). These pulses are initiated at puberty, under the control of kisspeptin neurons in the brain [15], persist at low levels throughout the estrous cycle, and frequency and amplitude is dependent on feedback from hormones produced by the ovary. GnRH also controls the release of luteinizing hormone (LH) from the pituitary. Estradiol and inhibin, produced by growing ovarian follicles, initially feedback on the hypothalamus and pituitary to decrease the release of FSH. Activin, a relative of inhibin in the transforming growth factor β (TGFβ) superfamily acts in an opposite manner, and is produced by small ovarian follicles to promote FSH release from the pituitary [16].

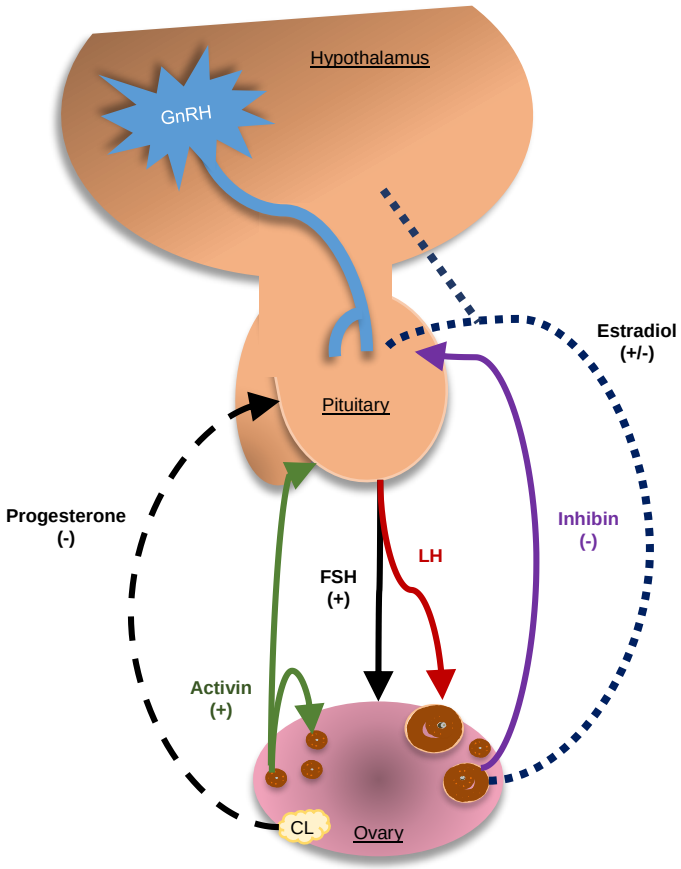

Figure 1.1: Overview of the hypothalamic-pituitary-ovarian axis. Modified from a combination of Senger [17], Durán-Pastén and Fiordelisio [18], and Gupta and Chia [19]

Once secretion of estradiol by dominant follicles reaches a certain threshold, it produces a positive feedback loop with the surge center of the hypothalamus, ultimately resulting in a peak of LH (considered Day 0 of the cycle) [20] which acts back on dominant follicles to stimulate ovulation [21]. Immediately following ovulation the site of ovulation is remodeled to become a corpus luteum (CL). Once the CL is established, it produces progesterone to maintain the pregnancy in a period called diestrus. Beyond acting on the myometrium to inhibit uterine contractions, and thereby allow the early embryo to develop [22], progesterone also acts to reduce GnRH pulse frequency, suppressing LH and FSH [23, 24] and subsequent follicle

growth. In species with maternal recognition of pregnancy, the period of diestrus is short, as the uterus will signal for luteolysis in the absence of an embryo [25]. In others, the corpus luteum persists regardless of pregnancy status in an ‘obligate diestrus’ or ‘non-pregnant luteal phase’. Once luteolysis occurs and the negative effect of progesterone is removed, FSH will promote the development of another cohort of follicles for the subsequent cycle. Alternatively, many seasonally-breeding species will experience a period of anestrus, or period of reproductive quiescence, following diestrus. During this period, no ovulation and little to no ovarian follicle development occurs.

For seasonal breeders, signals regarding day length (amount of light vs dark) are received by the eye, which are passed along via the suprachiasmatic nucleus in the hypothalamus, eventually to the β-adrenergic receptors in the pineal gland, which results in the downstream production of melatonin (See review: [26]). Melatonin is secreted at night, and depending on the species and its particular breeding season, acts to inhibit or release gonadotropins and prolactin. For example, the hamster has a short gestation and, to give birth in spring, requires ovarian activity to be resumed as spring approaches (i.e. days become longer). Therefore, short-duration melatonin promotes reproductive activity in this species. Conversely, species like the sheep with longer gestations are short-day breeders, and exposure to long duration of melatonin at night promotes reproductive activity,

2. In canids

Domestic dogs experience a reproductive cycle consisting of one week periods of proestrus and estrus, followed by a 2 month obligatory diestrus [27]. There is no known mechanism of luteolysis, as the CL is maintained regardless of pregnancy status. Diestrus is followed by a variably long (2-10 month) anestrus [28]. Most wild canids are seasonal breeders, therefore the anestrus period makes up the majority of the year [29]. The non-seasonal domestic dog experiences a range of anestrus durations, and generally ovulates 1-2 times annually. However, the variable duration and final termination of anestrus are not well understood, despite decades

of work investigating the traditional reproductive hormones (estradiol, progesterone, gonadotropins) to see which if any of them are drivers of this transition.

FSH is relatively high in anestrus dog serum [30], indicating an active pituitary. However, FSH alone does not appear to be responsible for follicle recruitment and the termination of anestrus. LH is also present in the circulation of anestrus dogs with a pulsatile release pattern. During late anestrus, the level of gonadotropins, especially FSH significantly increases [31] as the result of GnRH release from the hypothalamus [32], which is likely driven by circannual cycle-related signals and/or pheromones [30, 33]. Regarding the latter, there is evidence that both the Maned Wolf [34] and Island Fox [35] are induced ovulators, in that ovulation rarely occurs unless the female is housed with or near a male, suggesting some kind of pheromone cue may also be responsible for the resumption of estrus cyclicity during the breeding season. Recently, Albers-Wolthers et al have demonstrated that kisspeptin likely plays a role in estrus resumption in the dog, as infusion of kisspeptin into anestrus dogs resulted in LH and FSH production [36]. However, understanding of the seasonal regulation of this factor in the dog is not yet known.

The increase in FSH concentration during proestrus promotes the development of small antral follicles into the preovulatory stage. Dogs also experience preovulatory luteinization, likely in response to the increased pulsatility of LH during proestrus, which results in rising progesterone levels in circulation prior to ovulation which can be used to identify day of LH surge and ovulation [37, 38].

3. Status of ART: Control of estrus cyclicity

a. General

Control of estrus cyclicity has become common practice in the human and some production animals, including the cow. In women, oral contraceptives containing a combination of estrogen and progestins which suppressed FSH and the LH surge, respectively, were first developed over 50 years ago [39]. Protocols for the stimulation of ovulation have also become

commonplace in human fertility clinics over the past several decades. Stimulation is typically performed prior to *in vitro* fertilization, in order to maximize the numbers of oocytes that can be collected [40]. Similar protocols have been developed in the cow, to induce estrus. This is economically attractive for a number of reasons, including its ability to increase the number of breeding opportunities or embryo numbers of genetically valuable cows per year. However, in both cases variability in treatment response has been observed between individuals, particularly with regard to age and body condition [41]. Much interest has been focused on finding and assessing the value of various markers which might be able to predict an individual's response to ovarian stimulation therapies. Recently, anti-Müllerian hormone (AMH) has been identified as such a marker [42] and is being widely utilized in human fertility clinics.

AMH is a member of the TGFβ superfamily which was originally identified for its role in the regression of the Müllerian duct in male fetal development [43]. However, it has also been identified in preantral to small antral ovarian follicles [44] and shown to counteract the action of FSH [45] and inhibit primordial follicle recruitment [46]. Because it is produced primarily by follicles [47], it has become an attractive marker for those interested in assessing the reserve of follicles in the ovary [48]. Furthermore, as women with higher AMH have more growing follicles, it has also become a useful marker in anticipating ability to respond to ovarian stimulation therapies [49].

b. In canids

Many attempts have been made to develop estrus induction protocols for the dog, with varying results (See Review: [50]). Supplementation of exogenous FSH to stimulate follicle growth has resulted in 28.6% of treated bitches coming into estrus in one study [51]. Dopamine agonists, meant to induce estrus via suppressing prolactin and thereby increasing gonadotropin release [52], have successfully induced estrus in most studies. However, duration of treatment needed to induce estrus was found to be dependent on stage of anestrus, wherein bitches in late anestrus came into estrus on average two weeks sooner than those originally in early

anestrus at the start of treatment [53]. Perhaps one of the most popular methods has been the use of GnRH agonists, such as deslorelin. GnRH agonists work by stimulating GnRH release and therefore FSH and LH [54]. When it is effective, deslorelin has the advantage that it has very few acute side effects and does not require daily treatments [55]. It has been successfully utilized in domestic dogs [54], grey wolves [56], and maned wolves [57]. However, effectiveness of deslorelin is also dependent on the stage of reproductive cycle at the time of hormone treatment. One study showed 100% of anestrus dogs resumed estrus cyclicity when treated with deslorelin, whereas less than 50% of diestrus bitches ovulated with this intervention [58]. Furthermore, long-term treatment results in down-regulation of GnRH secretion and estrus suppression [59], and therefore these agonists are also being utilized as contraceptives [60]. In summary, although many different estrus induction techniques have been attempted in the dog, few have yielded consistent results, particularly when evaluating efficacy over different stages of anestrus or diestrus. It is not just a matter of stimulating or mimicking the pituitary, but rather some aspect of development or priming must occur over the course of diestrus and/or anestrus that makes a bitch more likely to respond to these interventions. However, lack of understanding of the endocrine drivers of the anestrus to estrus transition holds back our ability to develop improved ART for estrus induction.

Folliculogenesis to Ovulation

1. Overview

At or shortly after birth, the mammalian ovary is believed to contain all the primordial follicles that female will have for her lifetime [61]. A primordial follicle consists of a centralized oocyte surrounded by a single layer of flattened granulosa cells (Fig. 1.2). Initial recruitment or activation of follicles from the primordial pool begins shortly after follicles are formed [62]. In contrast, cyclic recruitment or activation of follicles from the primordial pool is begins at puberty (See Review: [63].). Although the exact mechanisms of this transition are not fully understood,

morphologically it consists of the granulosa cells obtaining a cuboidal shape. At this point the follicle is considered ‘primary’, containing a single layer of cuboidal granulosa, but these cells are also becoming proliferative. Formation of the zona pellucida, or translucent glycoprotein layer surrounding the oocyte as well as initial recruitment of theca cells from the ovarian stroma begins at this point. Secondary stage follicles have at least two layers of granulosa cells and, together with primary stage follicles, are considered “preantral”, as they have yet to develop the fluid filled cavity of their later-stage counterparts. Vascularization of the theca cell layer will begin in late preantral follicles [64], which likely supports further growth and later antrum formation.

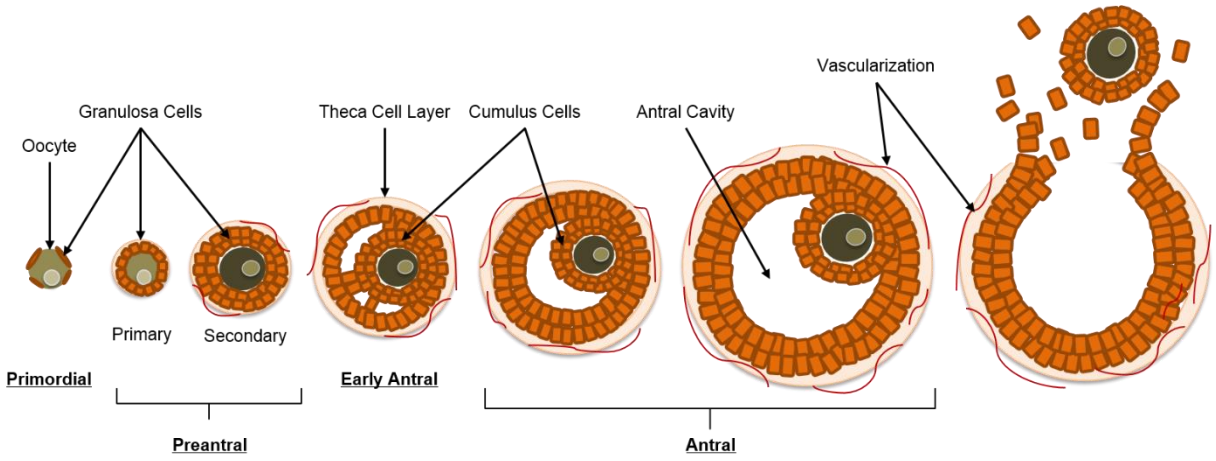

Figure 1.2: Folliculogenesis in the mammalian ovary. Adapted from Senger [17], with vascularization modifications based on [64].

The mechanism of antral cavity development is also not well understood. An increase in aquaporin expression/activity is thought to occur in granulosa cells, resulting in increased movement of fluid into the center of the follicle [65]. Another hypothesis is that an osmotic gradient, either via large molecules like versican being secreted [66], or accumulation of DNA from coordinated granulosa cell apoptosis is responsible/involved [67]. Antrum development begins with the formation of small fluid-filled pockets (early antral stage), which then coalesce

into a single cavity. The follicular fluid of the antral cavity is highly concentrated with hormones and growth factors, which are thought to contribute to the maturation of the oocyte.

At this point as well, granulosa cells around the oocyte communicate with the oocyte via trans-zonal projections, or extensions of the cells which traverse through the zona pellucida to form gap junctions with the oocyte [68]. Oocytes direct the development and differentiation of the granulosa cells into cumulus, as demonstrated by Eppig et a [69] in which oocytes from secondary follicles were transferred to primordial follicles, resulting in accelerated differentiation of the primordial follicle's somatic cells. Additionally, it has been shown that this communication is vital to the oocyte as well, as reduction in gap junctions between the cumulus cells and the oocyte results in poor granulosa cell development which, in turn, results in poor oocyte health and abnormal morphology [70]. The bi-directional communication via growth factors and other substrates between the granulosa cells and the oocyte is still being explored, but one example of this relationship is kit ligand. Kit ligand is a growth factor produced by granulosa cells, which acts on the oocyte to promote growth [71]. However, it has also been shown that the oocyte regulates the mRNA levels of kit ligand in the granulosa cells, thereby acting through somatic cells to control their own growth and development [72]. Further, cumulus and granulosa cells work together in the presence of gonadotropins to produce estradiol and progesterone [73], which are required for further follicle development and eventual ovulation.

During the follicular phase, a single follicle (or follicles, in the case of multiparous species) is selected to become dominant. Rapid growth and expansion occur, leading up to the LH surge. The LH surge is critical for ovulation in several ways, including increasing blood flow, and promoting the production of proteolytic enzymes which weaken the follicle wall [74]. Rising pressure from the fluid-filled antral cavity eventually causes a rupture in the weakened follicle wall, releasing the ovum. In many species, the ovulated oocyte has already resumed meiosis and therefore is mature/fertilizable by the time it reaches the oviduct. Work in the mouse has elucidated this mechanism, wherein cyclic GMP produced by the cumulus cells diffuses into the

oocyte via gap junctions to inhibit phosphodiesterase (PDE3A) activity. Inhibition of PDE3A stops the hydrolysis of cAMP needed for resumption of meiosis [75]. The LH surge decreases the cGMP produced while also closing the gap junctions between the oocyte and the cumulus cells, thereby allowing meiosis to resume.

2. In canids

Due to the anatomy of the dog ovary, which includes an all-encompassing bursa, information regarding the follicular dynamics on the surface of the ovary has been limited, although ultrasound data from England et al demonstrated the emergence of antral stage follicles (<3 mm) from the surface of the ovary beginning approximately two months and peaking two weeks prior to the LH surge [76]. Additionally, canid oocytes are ovulated at an immature stage compared with other species (germinal vesicle stage, or GV), and require 48 – 72 hours in the oviduct post-ovulation to complete nuclear maturation [77]. The mechanism of post-ovulatory oocyte maturation in the dog is not known.

An improved understanding of the mechanisms of folliculogenesis in the domestic dog is needed. Fortunately, the ready availability of dog ovarian tissue from routine ovariohysterectomies at veterinary hospitals and spay clinics makes for an accessible and inexpensive source of samples that is well representative of the larger population of domestic dogs to begin these investigations.

3. Status of ART: *In vitro* follicle culture

a. General

Live offspring have been produced using oocytes from primordial follicles which were grown and matured *in vitro* in the mouse [78]. However, the same success has not been reached with larger mammals [79, 80]. This is due in part to the longer duration of folliculogenesis in larger animals – over 100 days in the dog [81] and human [82], versus only 21 days in the mouse [83]. Further, the final follicle sizes of large mammals (4-13 mm and 17-20 mm for the dog [84] and human [82], respectively) are much larger than that of the mouse (0.5-0.6 mm) [82]. As result,

the two-dimensional (2D) follicle culture used for the mouse fails to maintain the structure of large mammalian follicles and the critical connections between granulosa/cumulus cells and the oocyte during the long-term culture needed for folliculogenesis in these species.

Recent advancements in biomaterials have provided some options for overcoming challenges associated with 2D systems. Alginate, a polysaccharide which cross-links when exposed to a divalent cation such as calcium, has been employed to culture ovarian follicles of several species including the mouse [85], human [86], non-human primate [87] and dog [88]. In the rhesus monkey, secondary-stage follicles cultured while encapsulated in an alginate hydrogel survived, increased in diameter, and produced steroids [87]. The benefit of systems such as this is that they provide a scaffold around the follicles to maintain 3D structure, and also prevent adherence of the follicle to the dish by containing somatic cells.

A second generation of this system is the Fibrin-Alginate Interpenetrating Network (FA-IPN). Fibrinogen polymerizes into fibrin when thrombin activates factor XIIIa [89]. This matrix is digested-away by proteases/enzymes secreted by the growing follicle. Use of this system in the mouse has yielded high rates of oocytes reaching MII after follicle culture [89, 90]. This system has also been utilized in a non-human primate, resulting in the first production of MII oocytes from cultured small antral baboon follicles [91].

b. In the domestic dog

Some progress has been made in the development of *in vitro* folliculogenesis systems for canids as well. Primordial follicles in ovarian cortical tissue have been cultured for up to one week, [92] although follicle morphology became compromised after three days in culture. Pre- and early antral-staged follicles cultured in a three-dimensional alginate hydrogel system were reported to have increased in diameter by 100% in the presence of high concentrations of FSH, but estradiol production by these follicles was not observed [88] suggesting a possible requirement of LH by these growing follicles for normal steroidogenesis. Serafim et al [93] found that isolated follicles 275.39 ± 6.55 μm diameter (which would be classified as “early antral”,

based on the metrics utilized in this dissertation [94]) grew on average to 439.8 ± 14.08 µm, or approximately 60% growth, over 18 days in a two-dimensional culture system with increasing concentrations of FSH supplementation. Additional antrum development was observed in over 80% of the follicles cultured, but significant oocyte extrusion was also noted in follicles treated with high levels of FSH. This phenomenon has been noted in the culture of follicles of other species [95], and may be due to a breakdown of connections between granulosa cells and the oocyte.

Based on the results of the previous studies, two hormones of interest have been identified; LH, for its role in steroidogenesis, and activin, for its potential role in maintaining trans-zonal projections. The role of LH in follicle steroidogenesis has been discussed previously, and supplementation of LH has also been shown to promote murine preantral follicle growth and antrum formation [96, 97]. In the goat, preantral follicles grown for 6 days (reaching the early-antral stage) in culture, then exposed to LH, had improved survival and ability of oocytes to resume meiosis; however, gametes from preantral follicles exposed to LH from the onset of culture had reduced capacity to resume meiosis [98]. It was hypothesized that this negative effect of LH on small follicles was the result of premature luteinization. Therefore, the effect of LH on dog follicle growth and development may be stage-specific as well.

Activin is a glycoprotein member of the transforming growth factor β (TGFβ) superfamily. There is evidence from several species, including the goat [99], pig [100], monkey [101], and human [102], that activin is produced by ovarian follicles, particularly those of the primary to preantral stage. Activin, when free from its inhibitory binding protein, follistatin, is thought to act both as an endocrine factor, signaling to the pituitary to promote FSH release, and as a paracrine/autocrine factor in the ovary, improving follicle growth [103] and antrum development [104, 105] as well as promoting survival [106] and maturation of the oocyte [102, 107]. Activin is thought to exert its effect through the Smad pathway after binding to cell surface receptors and triggering several downstream cascades [108-110] (Fig. 1.3). Previous research on activin has

primarily focused on its role in increasing sensitivity of follicles to FSH, which has been shown to operate through increasing transcription of FSH receptor mRNA [111, 112]. A recent *in vitro* study suggests that activin improves cumulus-oocyte communications by maintaining trans-zonal projections (TZPs) [113] and this, in turn, promotes oocyte health and antrum formation. This hypothesis is supported by studies on activin knock-out mice, in which ovarian follicles do not progress past the early antral stage [109].

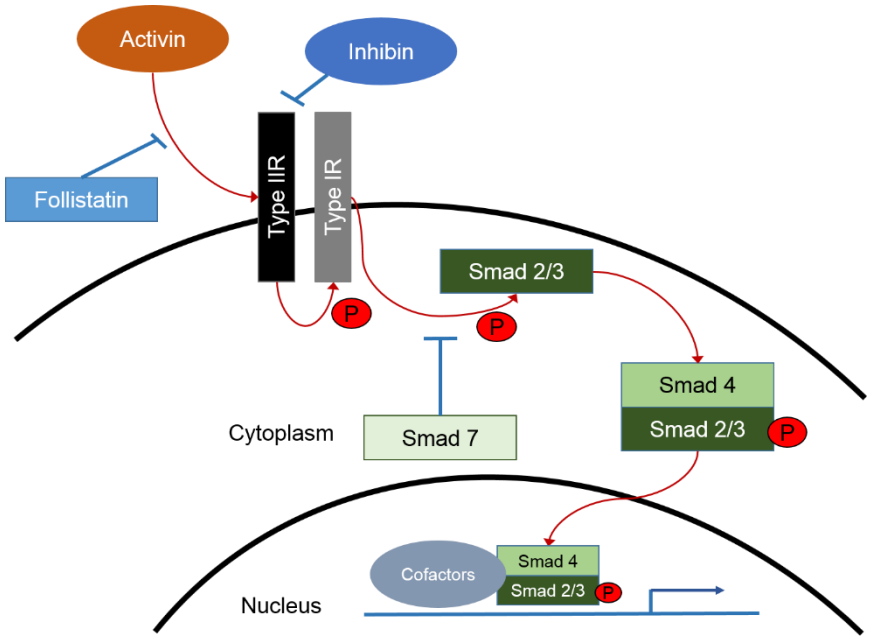

Figure 1.3: Signal transduction of the activin pathway. Adapted from Asa and Ezzat [114] and Wakefield and Hill [115].

Fertilization and Embryo Development

1. Overview

The sperm is made up of two main parts. First the head contains the nucleus, and is itself capped at the rostral end by a single vesicle known as the acrosome. This exocytotic vesicle contains enzymes to aid in sperm penetration into the egg, both through the surrounding cumulus cells as well as through the zona pellucida. The flagellum or tail is itself divided into two major parts, with the central axoneme running the full length. Closest to the head, the midpiece

contains mitochondria, which power motility to differing degrees depending on the species. Distal to that, and making up the majority of the length of the sperm, is the principal piece. This region contains a variety of signaling and metabolic enzymes, including those of the pathway of glycolysis which provides local production of ATP [116].

Ejaculated sperm are not immediately competent to fertilize an oocyte, and first must undergo a series of developmental events termed ‘capacitation’. Capacitation is the process by which a sperm becomes developmentally competent to fertilize [117], and is characterized by hyperactive motility and ability to undergo acrosome exocytosis. Once in the female reproductive tract, sperm are exposed to stimuli which promote bicarbonate and calcium entry into the sperm and efflux of sterols from the plasma membrane, both of which promote capacitation [118].

A mature, post-ovulatory, haploid oocyte is arrested at metaphase II. The interaction of the capacitated sperm with the cumulus cells surrounding the oocyte triggers acrosome exocytosis [119], and the release of enzymes to facilitate sperm penetration. Entry of the sperm triggers the cortical reaction in the oocyte, wherein enzymes are released which harden the zona pellucida as a block to polyspermy [120]. The oocyte also resumes meiosis and the haploid chromosomes of the sperm and egg fuse to produce a diploid zygote, which will then begin mitotic cell divisions [17]. Once a >16 cell embryo has developed, the embryo is considered a morula. Beyond this point, a fluid filled cavity will form in the embryo and the cells begin to differentiate into an inner cell mass, which will give rise to the fetus, and the trophoblast, which will become the placenta [17]. Eventually this blastocyst will hatch from the zona pellucida and begin to implant into the uterine wall, which will facilitate growth and development for the remainder of the pregnancy.

2. In the domestic dog

As previously stated, the mechanism of meiotic resumption in the ovulated oocyte is not understood for the domestic dog; however, canine sperm capacitation is believed to operate

similarly to that of other species [121]. Within about 24 hours post-ovulation, the oocyte has moved into the isthmus of the oviduct, where fertilization occurs after meiosis has completed [77]. The timeline of maturation and early embryo development is not consistent, even among oocytes observed simultaneously in the same bitch [77]. Further, the fertilization window for dog oocytes appears to be long, as viable pregnancies have been produced via intra-uterine AI even up to 10 days post LH surge [122]. Once fertilized, the dog embryo remains in the oviduct for approximately 11 days, reaching the morula stage prior to entry into the uterine horn [123, 124]. There, embryos continue to develop, hatching and attaching by around Day 21 post LH surge [125].

3. Status of ART: *In vitro* fertilization

a. General

Significant advances have been made in the development of embryo-based technologies in the past several decades. Live young have been produced via IVF in the laboratory mouse [126], cow [127], and the woman [128], among other species. Intra-cytoplasmic sperm injection, wherein a sperm is mechanically injected into a collected oocyte, has become commonplace in human fertility clinics as well. This method allows doctors to bypass sperm capacitation requirements, which is important for couples with male fertility issues.

b. In the domestic dog

Despite decades of research, there has never been successful production of live offspring by IVF in any species of canid. We have identified three potential reasons for these previous failures: non-ideal sperm capacitation conditions, poor quality of *in vitro* matured oocytes, and inadequate conditions (e.g. media, temperature, and gas) used for culture.

Although beyond the focus of this dissertation, evaluation and improvements to canine sperm capacitation medium were initiated as part of the IVF study. An *in vitro* culture medium was previously developed for dog capacitation [129]. However, this medium omitted magnesium

on the basis that supplementation delayed incidence of spontaneous acrosome exocytosis by several hours. Magnesium is an important co-factor for glycolytic enzymes, and has been shown to promote acrosome exocytosis via a Ca^{2+}-Mg^{2+}-ATPase in bull and ram spermatozoa [130]. Additionally, *in vivo* acrosome exocytosis is stimulated by progesterone and/or zona pellucida proteins [131], therefore evaluating a sperm's ability to undergo exocytosis induced by a physiological stimulant is more appropriate. Based on this, we evaluated the effect of magnesium supplementation to canine capacitation medium (CCM) on sperm motility, hyperactivation, tyrosine phosphorylation, and ability to undergo acrosome exocytosis in response to progesterone. Magnesium was found to have a beneficial effect on all metrics of sperm capacitation (Sylvester, Nagashima et al., manuscript in preparation), and therefore was included in the *in vitro* fertilization studies presented here.

The second potential reason for IVF failure is the quality of oocytes used. Dog IVF studies have historically utilized *in vitro*-matured (IVM) oocytes. These oocytes are derived from follicles of variable developmental stages out of the ovaries of dogs primarily in anestrus, and IVM generally results in low rates of successful resumption of meiosis. Although attempted protocols vary, some have utilized equine chorionic gonadotropin for its FSH and LH activity, and epidermal growth factor to promote development of dog oocytes to metaphase II [132, 133]. *In vivo*, sperm rarely penetrate the zona pellucida of oocytes prior to their reaching this stage [77]. It has also been shown that oocytes from antral follicles, particularly those larger than 2 mm diameter are significantly more successful (79.5 ± 10.9 compared to 16.9 ± 9.2% from those < 0.5 mm diameter) at reaching MII *in vitro* [132]. Although large follicles are more likely to be found in proestrus and estrus, when controlling for follicle size, no difference was found in the ability of oocytes from different stages of the reproductive cycle to reach MII [134]. However, success varies between studies, as another laboratory found that even when oocytes from preovulatory follicles were used, only 31.9% reached MII, although anestrus controls only yielded 12% MII after *in vitro* maturation [135]. Therefore it is likely that these previous attempts

(Table 1.1), which have only produced 1 blastocyst [136] and 3 morulae [137-139] out of hundreds of oocytes, failed due to poor quality or developmental capacity of oocytes that have been matured *in vitro*.

Table 1.1: Summary of results of select IVF studies utilizing *in vitro* matured oocytes [133, 136-138, 140, 141]

Study	Cleavage	≥ 8-Cell	Notes
Otoi et al 2000	14.3%	N/A	1 blastocyst produced
England et al 2001	2.2%	N/A	Implantation after transfer of ≤ 2-cell embryos
Songsasen et al 2002	8.2%	N/A	Early cleavage
Otoi et al 2004	28.3%	5.6%	1 morula produced
Hatoya et al 2006	4.3%	1.2%	1 morula produced
Saikhun et al 2007	33.6%	4.1%	No embryos > 8-16 cell produced

Lastly, the microenvironment to which the fertilizing oocyte or embryo is exposed is known to be critical to its successful development in many species. For example, in the hamster a block at the 1-cell stage after IVF existed for years until it was determined that glucose and phosphate in the culture medium were inhibiting embryo development [142]. On the other hand, *in vitro* development of bovine embryos required addition of amino acids to progress beyond the 8-cell block [143]. In most cases, the timing of the block coincides with embryonic genome activation, wherein the developing embryo must begin to produce its own RNA and proteins for continued development, rather than relying on maternally-inherited material [144, 145]. Although domestic dog embryo development rates are low to begin with, in previous studies there appears to be a decrease in embryos reaching the 8-cell stage (stage of embryonic genome activation in the dog [146]), with a maximum rate of 5.6% (Table 1.1)[137]. As the canine

embryo becomes fertilized and continues to develop in the oviduct until approximately Day 11 post LH surge [124], the microenvironment of the oviduct must be the basis for development of fertilization and culture media conditions. Recent oviductal fluid evaluation has revealed that progesterone levels are high in dog bursal and oviductal fluid following ovulation [147]. This work is complemented by progesterone receptor localization studies demonstrating an increase in receptors in this area during the periovulatory period [148, 149].

Summary

The domestic dog holds great promise as a research model for wild canid conservation efforts and human biomedical research. The ability to control dog reproduction would also be valuable for the development of contraceptives to curb overpopulation. However, our understanding of many of the mechanisms of dog reproduction is still lacking, including the drivers of estrus cyclicity, folliculogenesis, and fertilization. Each of these aspects are needed in order to fully realize the potential of the domestic dog as a research model. For example, understanding of the regulators of the transition from anestrus to estrus may allow us to develop methods for maternal genome rescue via *in vitro* follicle culture, by understanding the drivers of large, preovulatory follicle development. Furthermore, knowledge of this transition will inform estrus induction protocols. These would improve our ability to collect *in vivo* matured oocytes which will help us develop and optimize *in vitro* fertilization protocols. Having a successful and reliable IVF protocol will in turn help us develop *in vitro* maturation protocols in that IVF will give us a way to test the developmental competence of the oocytes being produced. The following chapters describe studies on folliculogenesis, the transition from anestrus to estrus, and fertilization in the domestic dog. These studies were completed with the goal of improving understanding of the underlying mechanisms of dog reproductive biology for future application to the development of assisted reproductive technologies for endangered canid conservation.

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CHAPTER TWO

Follicular Size/Stage and Gonadotropin Concentration Influence Alginate-Encapsulated *In Vitro* Growth and Survival of Pre- and Early-Antral Dog Follicles*

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Abstract

Understanding stage-specific requirements of mammalian folliculogenesis is limited in the domestic dog. This study examined the influences of two potential regulators of dog follicle growth and survival *in vitro*: 1) original stage of the follicle (preantral, ≤230 μm diameter versus early antral, >230–≤330 μm); and 2) concentration of follicle stimulating hormone (FSH) and/or luteinizing hormone (LH). After isolation and alginate-encapsulation, follicles were cultured in 0, 1, 10, or 100 μg/ml FSH and 0, 1, or 10 ng/ml LH for 20 d. Regardless of stage, FSH promoted growth, but LH did the same only in the absence of FSH. Production of estradiol-17β and progesterone was detectable, indicating theca cell activity. The greatest growth occurred in preantral (mean ± standard deviation, 61.4 ± 25.9%) versus antral (42.6 ± 20.3%) follicles, but neither developmental stage nor gonadotropin influenced survival. Antrum detection was minimal due, in part, to antral collapse, and oocytes displayed an increasingly pale appearance and chromatin degeneration over time. Results demonstrate that pre- and early antral stage dog follicles encapsulated in alginate grow significantly *in vitro*. However, because FSH and LH alone or in combination fail to promote antrum development, the next step is identifying factors that enhance antral expansion.

Introduction

There is minimal information on regulation of ovarian folliculogenesis in the domestic dog, a species that is challenging to study at the follicular level *in vivo*. The dog's anatomy restricts viewing the ovary in situ because of an all-encompassing ovarian bursa [150]. Females experience a protracted, but variable duration of proestrus (5–20 d) and then estrus (5–15 d) that occur sporadically only once or twice annually [27, 151, 152]. The bitch is also generally resistant to ovarian stimulation by exogenous gonadotropin treatments [50]. This is likely due to our lack of understanding of the endocrinologic drivers of anestrus termination/estrus resumption in the dog.

What we know is that FSH and LH are present and pulsatile in the circulation of the bitch during anestrus. Prior to the onset of proestrus, the level of gonadotropins, especially FSH, increases significantly (Kooistra et al. 1999) due to GnRH release from the hypothalamus [32] that, in turn, was stimulated possibly by pheromonal or circannual cycle-related signals [30, 33]. It is this chronically elevated FSH that promotes development of a cohort of small antral follicles to the preovulatory stage.

Our overall aim is to understand the complex dynamics of dog follicular growth and maturation through *in vitro* studies. To illustrate, it is known that the size of a given dog follicle influences the developmental ability of its resident oocyte. For example, only ~17% of oocytes recovered from follicles <0.5 mm in diameter have the capacity to achieve metaphase II (MII) in culture compared to nearly 80% of those from follicles >2 mm [132]. We are intrigued about what regulates (or limits) the growth and developmental competence of oocytes from small versus large follicles. Such information could lead to effectively controlling reproduction in the dog as well as wild Canidae relatives [14] where females can be in anestrus for most of the year [153-155]. One of our areas of focus is the vast pool of small, growing follicles that will never develop, ovulate, or contribute to reproduction, but rather are destined for atresia. To produce meiotically-competent oocytes from this significant resource will require an ability to grow individual follicles to a minimum, as-yet-undefined size, but likely of an antral stage.

Offspring have been produced from mouse oocytes recovered from cultured primordial and preantral stage follicles [78, 156]. However, murine methods have not been adapted successfully to achieve the same result in other mammals [6, 79], mostly because larger species are more complex in reproductive form and function. For example, a viable mouse oocyte is normally recoverable from a follicle 0.5 to 0.6 mm in diameter [82] after a developmental period of only 21 d [83]. By contrast, complete folliculogenesis in the dog may require 100 d in vivo [81], and oocytes are only competent to resume meiosis if recovered from follicles ≥2 mm diameter [132], ~four-fold larger than in the mouse. Three-dimensional (3D) systems relying on alginate

hydrogel have improved follicular culture in the mouse [157, 158], non-human primate [87], and human [86, 159], presumably because this approach assists in physically retaining follicular shape and cell-to-cell interactions. From earlier work in our laboratory [88], we determined that domestic dog preantral follicles (≤230 μm diameter) cultured in an alginate hydrogel and supported by FSH survived for 10 d, increased in diameter, and produced progesterone (P4), but not estradiol-17β (E2). However, none of these follicles developed antral cavities or reached the critical 2 mm size threshold to produce an oocyte capable of meiotic maturation *in vitro*. Furthermore, the negligible estradiol production suggested a misregulation or missing factor required for functional steroidogenesis in these cultured follicles.

These observations led us to speculate about the specific role of FSH and LH in regulating follicle and oocyte development in the dog. Specifically, we became interested in how developmental stage of the follicle influences its sensitivity to gonadotropin stimulation. For example, preantral murine follicles with two granulosa cell layers do not require FSH to survive or even sustain low levels of growth in culture [160]. By contrast, preantral follicles with multiple granulosa cell layers are FSH-dependent for both survival and advanced expansion. Further, oocyte competence is improved when FSH is present in these follicular cultures [161]. LH also is known, in the presence of FSH, to promote preantral follicle growth and survival, and enhance antrum formation in the mouse [96, 97].

As no such knowledge exists for the domestic dog, our objective was to thoroughly understand the need for, and sensitivity to, what are believed to be two key controllers of folliculogenesis, FSH and LH. Our hypotheses were that: 1) FSH is essential for growth and survival of both preantral and early antral stage follicles, and acts in a dose-dependent fashion *in vitro*; 2) the role of LH is more evident in later follicular stages, especially associated with the timing of antrum formation and expansion; and 3) it is possible to over-saturate the microenvironment with gonadotropins, especially LH, causing a detrimental effect on follicle morphology and viability. The study was enhanced by our ability to isolate and longitudinally

monitor individual dog follicles of two stages in a 3D microenvironment for follicular growth, steroidogenesis, and oocyte survival.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO) unless otherwise stated. Alginate (Pronova UP MVG [catalogue no. 4200106], FMC BioPolymers, Philadelphia, PA) was provided by Dr. Teresa Woodruff (Northwestern University, Chicago, IL).

Follicle Isolation, Encapsulation, and Culture

Paired ovaries from 19 dogs of various breeds (age, 4 mo, n = 3; >8 m to 1 yr, n = 8, >1 yr to 6 yr, n = 8) were recovered during routine ovariohysterectomy procedures conducted under general anesthesia at local veterinary clinics and spay clinics over 14 culture days. Immediately upon excision, gonads were immersed in a solution of 0.9% NaCl (catalogue no. S5886) containing 10 IU/ml of penicillin G sodium and 10 µg/ml streptomycin sulfate (catalogue no. P4333), and then transported to the laboratory on ice. Isolation of follicles began within 3 h and was completed within 6 h of original surgery. The cortical portion (~1 mm thick) containing small, growing follicles was dissected from each ovary's surface. These were sectioned into ~3 mm^2 pieces that then were exposed as a collective cohort to 0.7 Wünsch units/ml Liberase Blendzyme (product no: 05401119001; Roche Applied Science, Indianapolis, IN) in α-minimum essential medium (αMEM [catalogue no. 9142], Irvine Scientific, Irvine, CA) containing 3 mg/ml BSA (catalogue no. A3311), 2 mM glutamine (catalogue no. 59202C), 10 IU/ml of penicillin G and 10 µg/ml of streptomycin (referred to as 'collection medium'). This enzymatic digestion process was permitted over a 75 min period at 38.5˚C (which is the physiological temperature of the dog) in 5% CO_2 in air [162]. Liberase Blendzyme was used as a gentler alternative to collagenase, while still being a sufficiently robust enzymatic digestion method to free follicles from the dog's rigid ovarian cortex. Follicles having at least two layers of granulosa cells with a homogeneously-dark,

centralized, circular oocyte and intact basement membrane [88] were separated from surrounding cells using a 25 gauge needle. Theca cell presence was not assessed as part of the selection criteria. Using a Leitz DM-IL inverted microscope (Research Instrument Limited, Falmouth, Cornwall, UK) with a heated stage and optical micrometer, we made two measurements of follicle size, including the widest length and the diameter perpendicular to it. The average of these two metrics was calculated and reported as 'follicle diameter'. As our laboratory has previously evaluated the relationship between size and stage for domestic dog ovarian follicles in vivo (Songsasen et al. 2009), isolated follicles used for culture were categorized by one of two known stages: preantral (i.e., 100–230 μm diameter) or early antral (>230–≤330 μm).

A total of 335 follicles was isolated (preantral, n = 168; early antral, n = 167), most of which were recovered from pubertal dogs (8 mo – 1 yr old = 183 follicles; > 1 yr old = 137 follicles). Few follicles (n = 15) were collected from prepubertal (< 6 mo) ovaries. Follicles were individually encapsulated in 0.5% (w/v) alginate using methods adapted from Xu et al [163]. In brief, 2 to 3 μl of alginate was pipetted onto a polypropylene mesh (0.1 mm opening; McMaster-Carr, Atlanta, GA). Each follicle was transferred, via wiretrol (a glass capillary with a plunger that uses negative pressure to aspirate cells and medium; Drummond Scientific, Broomall, PA) into each alginate drop. Using forceps, the mesh was held droplet-side down over a plastic Petri dish containing calcium chloride (5 mM $CaCl_{2m}$ [catalogue no. C7902] and 14 mM NaCl [catalogue no. S5886]) solution for 10 s to allow drops to bead. The forceps were tapped gently against the edge of the dish so that alginate drops containing follicles detached from the mesh and fell into the calcium chloride solution. After allowing cross-linking for 2 min, each alginate-encapsulated follicle was washed once in collection medium before being transferred into an individual, pre-equilibrated 85 μl droplet of growth medium. The latter was comprised of αMEM containing 1 mg/ml fetuin (catalogue no. F2379), 3 mg/ml BSA, 10 □g/ml insulin, 5.5 □g/ml transferrin, and 5 ng/ml selenium (catalogue no. I3146) [88] fortified with (or without) specific gonadotropins at various doses

(according to our treatment protocols, see below). The growth medium droplet containing the alginate-encapsulated follicle was then overlaid with mineral oil.

Encapsulated follicles from each dog were assigned randomly to at least 3 (average = 9) of the 12 designed hormone groups consisting of four logarithmically increasing FSH (Folltropin V, BioNiche Animal Health, Athens, GA; porcine pituitary derived) concentrations (0, 1, 10, or 100 µg/ml or 0, 0.002, 0.018, and 0.175 IU, respectively) and three LH (Lutrophin V, BioNiche Animal Health; porcine pituitary derived) dosages (Armour standard 0, 1, or 10 ng/ml) (a 3 x 4 factorial design; n = 19–27 follicles/group, approximately half being preantral and half being antral in size). These concentrations were chosen to cover both the known physiological range of these hormones in circulation (0.2–4.0 µg/ml and 0.4–40 ng/ml for FSH and LH, respectively; [27] as well as the higher levels used in a previous study by our laboratory [88]. Furthermore, because it has been shown that alginate may limit access to hormones in the culture medium (Heise et al 2005), it was important to include the supra-physiological concentration in the present study. The culture environment was at a constant 38.5˚C in a humidified atmosphere of 5% CO_2 in air. Incubation occurred over 20 consecutive days, and the diameter of each follicle was measured every 48 h. On each measurement day, a given follicle also was assessed for 'survival', the opposite of 'degeneration' [87], which was declared when there was: 1) a continual decrease in diameter over at least two previous observation points; 2) evidence of oocyte deterioration (fracture, non-homogeneous discoloration, or cellular extrusion); or 3) granulosa cell fragmentation. At the time of each size and morphological assessment (every 48 h), 50 µl of medium (henceforth referred to as 'spent medium') was aspirated from each culture droplet and maintained at -80°C until analyzed for E2 and P4 concentrations (see below). This volume was immediately replaced with fresh growth medium so environment and volume remained constant.

Follicle and Oocyte Viability Analysis

On Day 20 (Day 0 = day that follicle was placed in culture) each follicle was freed from its alginate capsule by replacing each growth medium droplet with 80 µl of 3 mg/ml alginate lyase

(catalogue no, A1603) in collection medium for 30 min. Then four to five randomly-chosen follicles (pooled based on gonadotropin/size treatment group, n_{total} = 57) were assessed for follicle and oocyte viability using ethidium homodimer-1/calcein AM dyes (catalogue no. L3224; Molecular Probes, Eugene, OR) [164]. In this dual-staining process, the red-fluorescent ethidium homodimer-1 reflects membrane integrity and will not penetrate an intact/viable membrane. Conversely, calcein readily permeates membranes, but only expresses green fluorescence if cleaved by active intracellular esterases. The number of membrane intact and live oocytes was determined via green fluorescence of granulosa cells and the oocyte using epiflourescence microscopy.

Oocytes (n = 67) from the remaining isolated follicles were pooled and then denuded using a hypotonic 1% sodium citrate solution (dissolved in αMEM medium 140 mOsm), which has been speculated to loosen the connection between cumulus cells and the oocytes, facilitating disassociation during gentle mouth-pipetting [165]. This technique has been routinely used to removing cumulus cells of bovine oocytes (Gordon, 1994). Oocyte diameter was then assessed under an inverted microscope and the oocytes fixed in 4% paraformaldehyde and stored at 4˚C for 12 h before placement into PBS for at least 12 h. Nuclear status assessments were made by incubating fixed oocytes in 20 µg/ml Hoechst 33342 for 20 min followed by evaluation under fluorescence microscopy [166].

Hormone Production Analysis

Spent medium from six cultures were assessed for E2 and P4 production by follicles in each of the 12 treatment groups. Spent medium was combined on consecutive collection intervals (i.e., Days 2 + 4, 6 + 8, 10 + 12, 14 + 16 and 18 + 20) to obtain adequate volume for the E2 and P4 radioimmunoassays. This was necessary because some follicles died as the study progressed, gradually reducing the spent medium volume available for evaluation, especially in the later days of the investigation. Spent medium was shipped frozen to the Endocrine Technology and Support Laboratory of the Oregon National Primate Research Center/Oregon

Health & Science University. This facility has the highly sensitive assays required to assess hormonal concentrations produced from only limited cohorts of cultured follicles (n = 1–8). Even then, it was necessary to eliminate the Days 18 + 20 time point of culture for evaluating hormones, as few follicles survived *in vitro* for 20 d. E2 and P4 concentrations were measured in spent medium by radioimmunoassay after ether extraction and purification via column chromatography [167]. Assay sensitivity for E2 and P4 was ~3 and 5 pg/tube, respectively, with inter- and intra-assay variations of <10 and 15%, respectively.

Statistical Analysis

Follicles that failed to survive by Day 2 of culture (n = 46) were considered to have had an undetectable, inherent defect prior to incubation onset or resulting from the encapsulation process and, therefore, were excluded from further analyses. Growth data (the percentage diameter increase from the size measured on Day 0) were log transformed to achieve normality prior to statistical analyses using a mixed-effect ANOVA model (random effects = 'culture', fixed effects = [FSH] + [LH] + [FSH] x [LH] + original size/stage + [FSH] x original size/stage + [LH] x original size/stage + [FSH] x [LH] x original size/stage) and a post-hoc Tukey-Kramer HSD test for significance (JMP 9.0 software, Cary, NC). Results were expressed as means $\pm$ standard deviation of transformed data, as these were the data used in our statistical models. Hormonal data were analyzed based on day and treatment group using the nonparametric Wilcoxon test (Mann-Whitney U). Follicle survival over the culture duration was evaluated using Cox proportional hazards in R 3.0.1 [168].

Results

Follicle diameters on Day 0 and 20 for each culture treatment are shown in Table 2.1. Overall, follicles of both developmental stages (preantral and early antral) grew ($P < 0.001$) over the 20 d culture period (mean ± standard deviation, 61.4 ± 25.9 and 42.6 ± 20.3% average increase in diameter, respectively) regardless of hormone treatment group. Nonetheless, the final

size reached by cultured preantral follicles was less ($P < 0.001$) than achieved by the early antral counterparts (preantral stage, from 206.1 ± 17.9 to 333.75 ± 49.8 µm; early antral stage, from 271.3 ± 27.2 to 386.7 ± 66.8 µm).

Treatment		Preantral Follicles			Early Antral Follicles		
FSH (µg/ml)	LH (ng/ml)	n	Initial Diameter	Final Diameter	n	Initial Diameter	Final Diameter
0	0	6	208.0 ± 25.3	300.0	17	262.3 ±28.6	345.5 ± 53.7
	1	14	208.6 ± 15.5	292.0 ± 54.7	9	274.2 ± 32.1	346.6±72.2
	10	13	206.7 ±18.9	352.0 ± 34.5	10	278.2 ± 22.35	385.2±16.5
1	0	10	200.0 ±18.2	326.8 ± 44.5	17	261.8 ± 20.4	355.2 ± 43.1
	1	13	204.2± 17.5	348.3 ± 35.9	11	264.4 ± 24.1	365.1 ± 11.9
	10	13	206.9 ± 17.2	335.0 ± 45.2	13	265.5 ± 27.1	424.0 ± 74.1
10	0	15	210.3 ± 14.5	316.0 ± 67.2	9	279.7 ± 23.5	409.3 ± 51.6
	1	11	212.6 ± 11.8	382.5 ± 53.0	14	276.5 ± 24.6	405.5 ± 49.4
	10	7	204.6 ± 19.6	402.0	12	279.1 ± 39.7	428.9 ± 150.3
100	0	11	207.4 ± 21/1	348.5 ± 66.5	12	268.8 ± 17.9	405.6 ± 54.2
	1	11	203.4 ± 23.9	341.3 ± 42.0	15	273.4 ± 20.7	397.9 ± 60.6
	10	13	199.6 ± 18.7	330.2 ± 48.5	13	281.5 ± 32.0	390.7 ± 29.6

Table 2.1: Initial and final diameters of follicles isolated at the preantral and early antral stage, cultured in FSH (0, 1, 10 and 100 µg/ml) and LH (0, 1, or 10 ng/ml)

Each follicle group, regardless of gonadotropin supplementation, increased in size ($P < 0.05$) over the 20 d culture interval. However, there was an influence of gonadotropin dose. For example, follicles in the presence of 10 µg/ml FSH grew from a mean diameter of 245.0 ± 41.6 to 390.3 ± 94.1 µm (an average of 51.2 ± 28.1% growth) compared to from 240.1 ± 40.2 to 341.2 ± 50.3 µm (40.6 ± 19.8% growth) for those cultured without FSH ($P < 0.05$). In absence of FSH, the highest LH concentration (10 ng/ml) restored growth to FSH-presence levels (Fig. 2.1). Follicles with no gonadotropin support (control) or only minimal LH support (1 ng/ml) increased 32.9 ± 10.8% and 32.0 ± 20.0%, respectively, above original size at isolation, which was less ($P < 0.01$) than the 56.9 ± 16.6% increase with the 10 ng/ml LH treatment. The average largest size follicles

(425.5 ± 139.4 µm) by Day 20 occurred in the presence of 10 µg/ml FSH plus 10 ng/ml LH. Contrary to our hypothesis, oversaturation of follicles treated with the highest concentrations of hormones did not occur. Follicles cultured in 100 µg/ml FSH and 10 ng/ml LH did not experience reduced growth or survival by Day 20 compared to lower dosage treatments ($P > 0.05$).

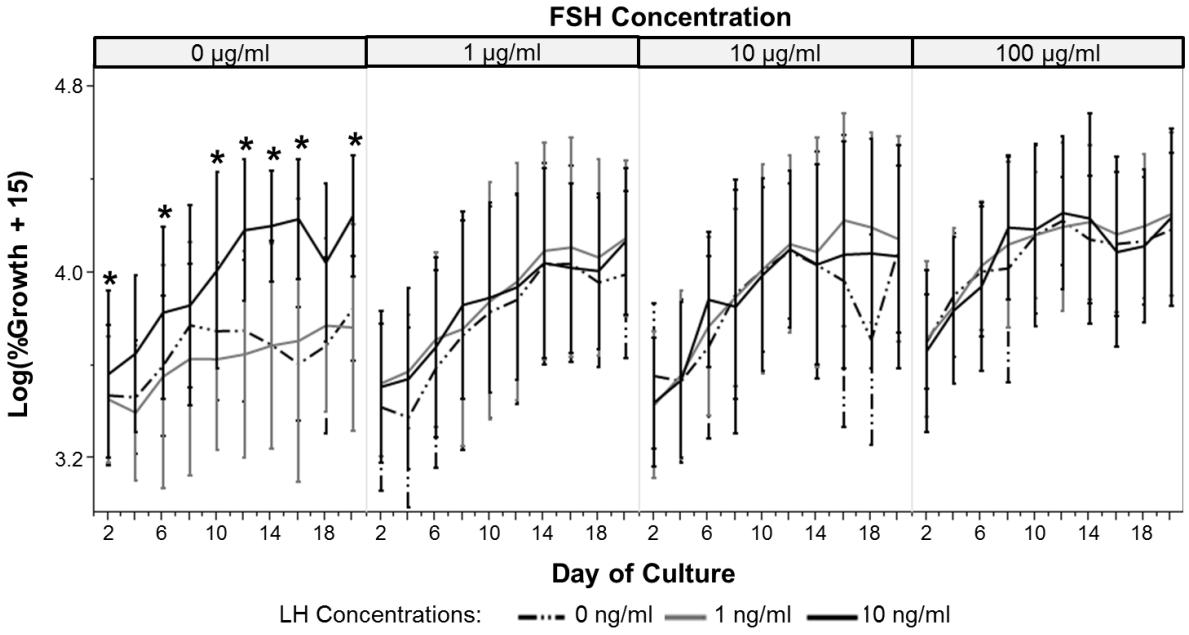


Figure 2.1: Growth over the culture duration (displayed as mean ± standard deviation of log-transformed data) of dog follicles exposed to different FSH and LH treatments. The asterisks (*) in the 0 µg/ml FSH group indicate point-in-time differences ($P < 0.05$) between the 1 ng/ml LH treatment and 0 and 10 ng/ml groups, demonstrating the restorative impact of this LH concentration in the absence of FSH.

Our growth model also revealed significant interactions between original follicle stage and FSH as well as FSH x LH. For example, FSH dose-dependently improved follicular growth (Fig. 2.1), but the impact was more pronounced in preantral compared to early antral follicles (Fig. 2.2). The interaction between FSH and LH also was more marked in preantral follicles, which responded with greater growth than early antral counterparts in low concentrations of both FSH

and LH (1 ng/ml LH and 1 or 10 µg/ml FSH) (Fig. 2.2). Neither gonadotropin, follicle stage, nor their interactions influenced ($P > 0.05$) follicular survival *in vitro*.

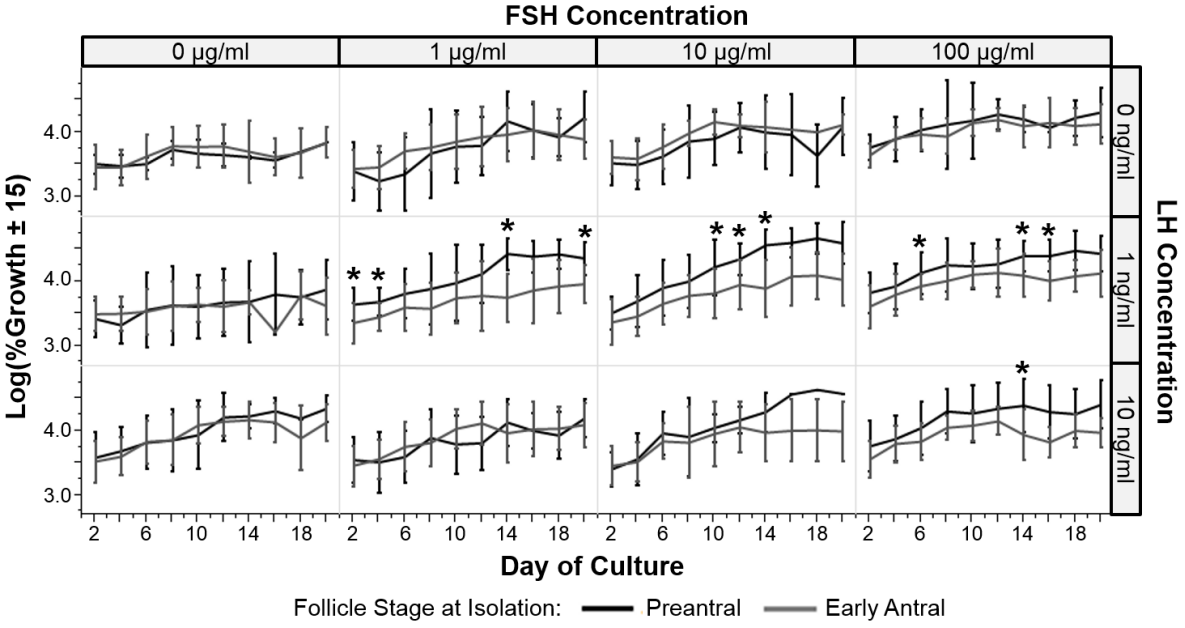
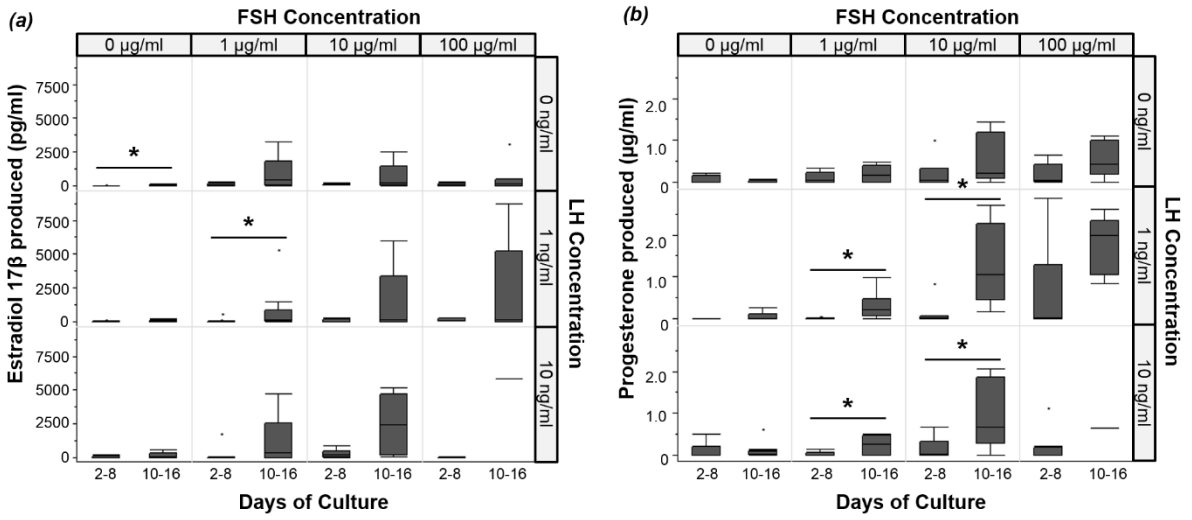


Figure 2.2: Interaction among FSH, LH, and original stage at isolation of dog follicles over time (displayed as log-transformed mean ± standard deviation). Asterisks (*) in each FSH panel indicate point-in-time differences ($P < 0.05$) between preantral and early antral stage groups for a given LH concentration.

It was possible to detect *in vitro* production of E2 and P4, although concentrations were initially minimal and became measurable and informative by Days 10 + 12 of incubation (Fig. 2.3). Specifically, mean E2 production rose ($P < 0.01$) from 47 pg/ml at this time to >1000 pg/ml by Days 14 + 16, although there was marked variability (range, 2-8734 pg/ml). Mean concentration of P4 production *in vitro* increased ($P < 0.01$) 10 fold from Day 2 + 4 of culture to Days 14 + 16. In the presence of LH and low levels of FSH, there was significantly more P4 produced in the second half of culture ($P < 0.05$, Fig. 2.3).



(c)

FSH (µg/ml)	0			1			10			100		
LH (ng/ml)	0	1	10	0	1	10	0	1	10	0	1	10
Days 2 - 8	43	41	36	45	50	38	38	42	31	35	27	22
Days 10 - 16	30	23	31	28	31	28	19	29	24	15	20	1

Figure 2.3: Box and whisker plots of production of *(a)* E2 and *(b)* P4 production according to FSH and LH treatments. To have adequate sample volume for assay and statistical evaluation, aliquots were combined for the first half (Days 2-8) and second half (Days 10-16) of culture. Hormone production generally increased after >10 d culture. For progesterone, this increase was significant ($P < 0.05$) in follicles treated with LH in the presence of low levels of FSH. *(c)* Indicates numbers of follicles contributing to hormone production results for each treatment group and culture period combination.

Typical follicular morphology and morphometrics over the 20 d culture are depicted in Figure 2.4. These are examples of a 220 µm diameter preantral (Fig. 2.4a) and 280 µm diameter early antral stage follicle (Fig. 2.4b) which advanced to 267 and 320 µm, respectively (Fig. 2.4c, d) over 6 d. Although the follicles were growing, the oocytes gradually took on a washed-out, pale appearance (Fig. 2.4c, d) that contrasted to the normally dark cytoplasm of freshly-collected oocytes (Fig. 2.4c, e, from 280 and 310 µm size-matched intraovarian follicle for images 2.4b and 5d, respectively). Evaluation of oocyte diameters (n = 46) from the start of culture (average 87.1

± 15.9 µm) to day 20 of culture (125.8 ± 18.7 µm) revealed significant growth ($P < 0.05$, Fig. 2.5a). Sample size for oocyte evaluation was smaller than that of nuclear staining as not all oocyte/zona pellucida diameters could be accurately measured within their follicles at the time of isolation (0 d). Of the total of 56 follicles examined for viability on day 20 using ethidium homodimer-1/calcein staining, 78.5% were living (Fig. 2.5b). However, of the 67 oocytes recovered from follicles incubated for 20 d, 32.8% expressed evidence of chromatin derangement and/or degeneration, with the remainder demonstrating moderate cytoplasmic and nuclear fracture (Fig. 2.5c). It was impossible to assess the incidence of antral cavity development because, when noted, small cavities had collapsed by the next observation point (48 hr later). We observed oocyte extrusion (expressed as an oocyte breaking through the follicular wall) in 12.4% of the cultured preantral stage follicles, but in only 4.6% of the early antral counterparts ($P < 0.01$).

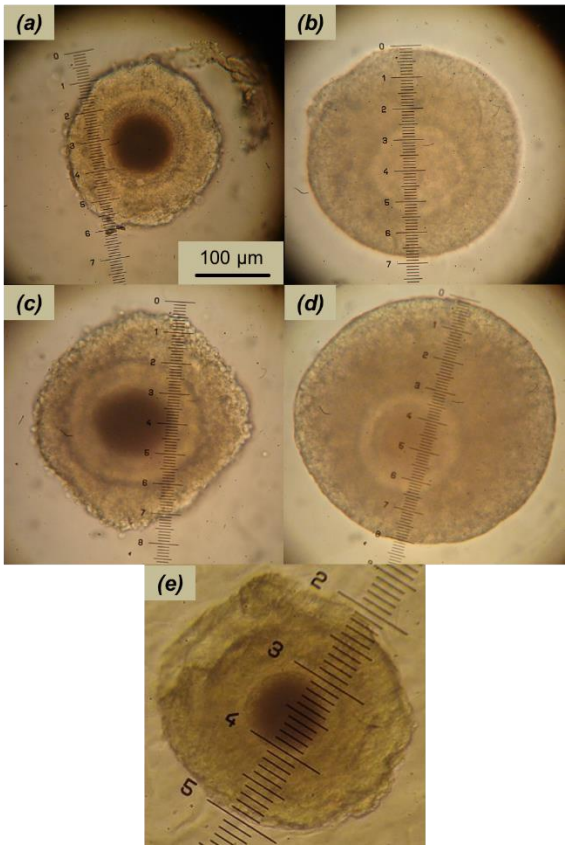


Figure 2.4: Photographs of *(a)* a freshly-isolated, preantral follicle (220 µm diameter); *(b)* the same follicle at 6 d of *in vitro* culture, revealing eventual growth to 268 µm and the increasingly pale appearance of the internal oocyte; *(c)* a freshly-isolated early antral follicle (280 µm) that serves as a size-matched control for the follicle in panel *b,* but was also cultured for 6 d and then imaged in *(d)* at 320 µm diameter*; (e)* a freshly-excised, size-matched (310 µm) control follicle for panel *d* displaying a typically dark, lipid-rich oocyte.

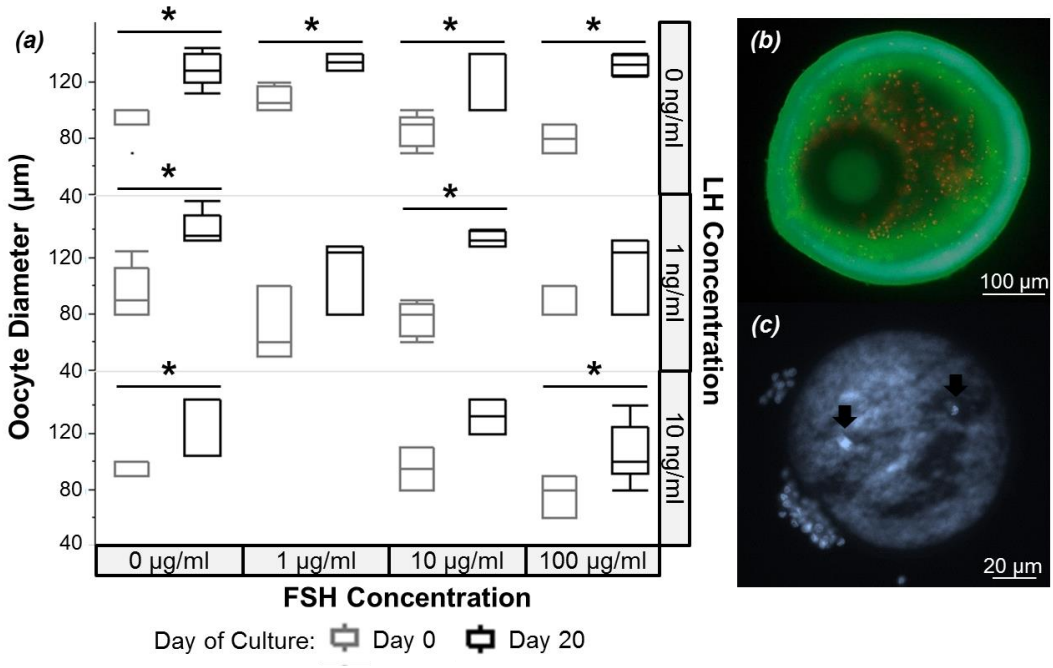


Figure 2.5: Box-and-whisker graph of *(a)* oocyte diameters (including zona pellucida) at initial follicle isolation (day 0) and after 20 d culture for each treatment group. Asterisks (*) represent significant differences ($P < 0.05$) between culture days for a given hormone condition. Images of *(b)* an antral follicle at the end of 20 d of culture stained with ethidium homodimer-1/calcein, and *(c)* a Hoechst 33342-stained oocyte after the 20 d follicle culture with fragmented cytoplasm and arrows indicating broken chromatin.

Discussion

Although the aim of follicular culture is to grow intraovarian follicles to a stage allowing the recovery of healthy, fertilizable oocytes (as has been achieved in the mouse [78, 156]); the prerequisite steps require detailed knowledge on regulation of folliculogenesis. To date, such information is limited on non-murine models, and we can assume that the challenges will be significant for a complex animal like the dog that has unusual reproductive norms. For example, protracted anestrus, sporadic onsets of ovarian activity, and resistance to exogenous gonadotropins affirm that dogs, and perhaps other canids, have atypical modes of folliculogenic

control. Thus, the present study was conducted to investigate the influence of two likely key factors, one being follicular stage (preantral versus early antral) and the other the gonadotropins FSH and LH on follicle growth and viability *in vitro*. We were particularly interested in the influence of gonadotropin dosage or combination on the development of these two different follicle types within a three-dimensional culture system. In this regard, we also examined the suitability of this microenvironment for antrum and oocyte development.

This approach allowed us to make four significant discoveries. First, FSH was important for follicular growth *in vitro*, although not necessarily linked to ultimate survival. Second, both preantral and early antral stage follicles advanced in size significantly in all treatment groups, with the greatest proportional growth occurring in the smaller-size cohort. Dog preantral follicles had the capacity to grow and respond to exogenous gonadotropins *in vitro*, but did not reach the size of their early antral counterparts, an inability likely due to an inadequate culture system. Third, we determined that, while clear growth occurred in early antral follicles *in vitro*, enlargement of the early antral cavity did not occur. Finally, although dog follicles excised from the ovarian cortex increased in size and produced estrogen and progesterone in the described system, these metrics did not equate with production of healthy oocytes, as oocytes evaluated from cultured follicles grew but experienced both abnormal cellular appearance and chromatin disruptions.

Because we took advantage of ovaries made available from routine spay/neuter procedures at local clinics, it was not possible to control the results on the basis of dog breed. Therefore our samples represented a genetically diverse population. We did collect multiple ovarian pairs from certain breeds (i.e., Chihuahua, German shepherd, golden retriever), but there was no significant difference in average numbers or morphology of follicles recovered. More important than dog breed were the notable benefits of recovering follicles from bitches that were $\geq$ 6 mo of age. These females were much more likely to provide follicles with oocytes that were homogenously-dark, consistent with normal lipid deposition in a growing dog oocyte [169]. By contrast, few follicles from ovaries excised from donors < 6 mo of age met this basic criterion.

Furthermore, in two of the three prepubertal donors, all recovered follicles either failed to grow at all or quickly extruded the oocytes in culture.

The finding that FSH enhanced growth of dog follicles *in vitro* was consistent with analogous observations in the mouse [160, 161], cow [170], nonhuman primate [171], and previous work by our laboratory on mixed-stage dog follicles [88]. The purported mechanism is that FSH binds to its G-protein coupled, transmembrane receptor to promote cell proliferation and steroidogenesis in granulosa cells [172]. In contrast to observing the consistent FSH effect, the influence of LH on ovarian follicles has been highly species-specific. In the mouse, adding LH to FSH-supplemented culture medium improves growth and survival of preantral follicles having two granulosa cell layers [96, 97]. *In vitro* cultured goat [98] and human [173] follicles exposed to FSH also benefit from supplemental LH with greater growth and antrum development. However, cow follicles exposed to LH alone (in absence of FSH) actually decrease in diameter over a 7 d culture interval [174]. By contrast, LH alone has a moderate stimulating effect on human preantral follicle growth and antrum development *in vitro* [173]. The same effect of LH in the absence of FSH was observed in our study for similar stage dog follicles, but with no added developmental benefits to *in vitro* folliculogenesis when exogenous FSH was present. Although LH pulses have been measured in circulation during mid- and late anestrus in the dog [31], the biological relevance of these brief bursts may be related to their episodic nature. By contrast, our *in vitro* culture system exposed dog follicles to chronic, elevated LH, which could have been contraindicated. For example, exogenous LH added to cultured rat preantral follicles has been discovered to down-regulate FSH receptor mRNA [175]. It also was possible that the positive effect of LH alone for enhancing dog follicle growth was related to weak cross-reactivity to FSH-like function. Although LH and FSH are highly-specific for their respective homologous receptors (LHR and FSHR) [176], heterologous binding and bio-assays across species have demonstrated striking variation [177, 178]. For example, purified turkey LH is equally potent to turkey FSH in binding chicken as well as reptile FSH receptor preparations [178]. Although the source of LH for our study was porcine-

derived, it may have bound to canine FSHR, especially at the highest concentrations. This may also address the lack of observable hormone oversaturation in our system, wherein follicles treated with the highest concentrations of FSH and LH did not experience the hypothesized reduction in growth and survivability. The porcine-FSH utilized in this study, even at 100 µg/ml chosen to oversaturate, may not have been specific enough to canine FSHR to truly oversupply the follicles.

In an initial investigation into the feasibility of culturing alginate-encapsulated dog follicles, we compared equine chorionic gonadotropin (eCG, which has both FSH- and LH-like effects; [179] versus purified (porcine) FSH over a 10 d incubation period [88]. Findings helped guide the present experimental design as E2 production occurred *in vitro* with eCG, but not FSH, strongly suggesting that supplemental LH probably was essential in the dog system, especially for steroidogenesis. In the current study, we detected both E2 and P4 in the culture medium, confirming that these cultured, early stage follicles had active theca cells, and that production of both steroids tended to increase with increasing FSH dose, a finding that may have become significant in the face of higher sample sizes. Like in the mouse [160], FSH absence resulted in a near-loss of progesterone synthesis. LH promoted P4 secretion in the latter half of culture, however concentrations were highly variable. This could have been related to a limited sample size and/or follicle quality variability, especially as culture progressed and some follicles died while others were sustained for various intervals before dying. The variation in P4 data also may have been associated with the higher LH concentrations provoking premature luteinization of granulosa cells in some follicles, but not others. The dog follicle is well known for its *in vivo* sensitivity to LH that results in preovulatory luteinization, a phenomenon essential to early progesterone secretion to facilitate onset of sexual receptivity [150, 152]. Lack of robust steroidogenesis also may have been due to a lack of, or immaturity in, the machinery of E2 and P4 production. Alternatively, use of mineral oil to suspend the culture droplets may have reduced the amount of these hormones present in culture medium, as steroids are notably lipophilic [180].

Increases in steroid hormone concentrations in the spent medium occurred slowly and were not elevated substantially until ~10 d of culture. In our previous investigation [88], we already had terminated the experiment by this time, so new findings here indicated that dog theca cells required more than 1 wk in this *in vitro* environment for the theca cells to proliferate and undergo steroidogenesis. The exact source of theca cells is not yet known, although the origin may be via recruitment or differentiation from the ovarian stroma when follicles possess multiple granulosa cell layers [181], possibly from a population of thecal stem cells [182]. Isolated sheep [183] and pig [184] theca cells recovered from antral follicles are able to produce significant amounts of androstenedione within 48 h culture in the presence of LH and insulin. Enzymatic digestion may have resulted in different numbers of theca cells associated with the individual follicles, resulting in variable hormone production, although Liberase was used (as an alternative to harsher collagenase) to obtain more morphologically-normal follicles [162]. The dog follicles in our study likely were on the cusp of the transition to the antral stage with just enough theca cells to allow modest steroidogenesis. It also was possible that these variations in steroid production were due to age and/or reproductive status differences among donors. For example, we have previously shown that expression of fibroblast growth factor (FGF) 2 and 7, thought to play a role in follicle development and steroidogenesis [185, 186], is influenced by time of the reproductive cycle in the bitch [94]. Specifically, FGF2 expresses in granulosa cells of secondary follicles in all stages of the reproductive cycle except anestrus. FGF7 is localized in both granulosa and theca cells of secondary follicles at all reproductive stages and in only the theca layer of antral follicles during proestrus and estrus. While age and/or reproductive stage may have influenced steroid production, our limited sample size does not allow us to draw further conclusions.

When there was morphological evidence of antral cavity expansion in these cultured dog follicles the effect was short-lived and these spaces collapsed by the next 48 h observation point. One possible limiting factor to antral expansion was the rigidity of the alginate hydrogel which, although useful for maintaining 3D follicular structure, may have actually suppressed overall

follicle growth. Studies by Woodruff and colleagues have determined that a less-dense alginate hydrogel concentration (0.25%) promotes growth, survival, and oocyte developmental competence in cultured mouse follicles [157, 163]. We had determined in previous comparative studies [88, 187] that a 0.5% concentration seemed best for dog follicles, largely as there is a difference in ovarian cortex rigidity compared to the mouse. However, this higher alginate-hydrogel density may, in fact, be limiting dog follicle growth and the goal of reaching a size consistent with producing a viable oocyte. One possible way of circumventing this issue might be derived from exciting advancements by Shea and colleagues in developing 'dynamic culture' systems that include a fibrin-alginate interpenetrating network (FA-IPN) [89], as well as hydrogels with matrix metalloproteinase-sensitive cross-linking peptides [188]. The concept here is that the follicle modifies its own microenvironment as it develops. A combination of alginate and fibrin comprises the FA-IPN complex, the latter of which is digestible by proteases secreted by the growing follicle, thereby softening the hydrogel and allowing for further follicle expansion via the usual modes of increased granulosa cell proliferation, cell growth, and/or antrum enlargement. The FA-IPN system has increased proportions of secondary-stage mouse follicles forming antral cavities and oocytes capable of achieving meiosis after culture [189]. Therefore, it seems logical that a next step priority for advancing dog follicle culture should be exploring a system like FA-IPN that more readily (and naturally) permits structural expansion and, thereby, more functional normality *in vitro*.

The phenomenon of follicular antrum formation and expansion remains relatively understudied in mammals. Recent *in vitro* studies have shown that supplementation of the protein hormone activin promotes bovine and human follicular antrum development and oocyte health [105, 113], possibly through the maintenance of trans-zonal projections between granulosa cells and the oocyte. Thus, future studies should investigate the roles of this growth factor on in vitro development of dog follicles and oocytes.

By the end of our 20 d culture, ethidium homodimer-1/calcein staining revealed viable granulosa cells and oocytes as well as a significant increase in oocyte diameter. The diameters reached by these oocytes were consistent with what has been observed in vivo in estrous dogs (range = 86.2 to 131.5 µm diameter; [77]. While this result was promising, nuclear evaluations of oocytes at this time revealed heterogeneous cytoplasm and fragmented chromatin. This is a common observation in cultured follicles from the domestic dog, where >40% of oocytes have been classified as degenerate within 72 h of incubation onset [190, 191]. The dark appearance of the dog oocyte is a cytoplasmic feature attributed to high lipid content [169, 192], which in the case of our cultured follicles, became pale and washed out over time. This observation likely reflected a failure of the system to meet the metabolic needs of the oocyte and forcing oxidation of endogenous lipid stores to generate energy for growth and survival. Our observations of occasional oocyte extrusions from these immature follicles has been described previously in cultures of goat [193], human [95], and mouse follicles [194]. This may be the result of an inability to establish trans-zonal projections between the oocyte and the granulosa cells, the latter often being absent on the extruded oocytes [95]. Our early findings with activin also may be helpful in better understanding the role of trans-zonal projections on energy substrate channeling and lipid balance in dog follicles.

In summary, our findings revealed that *in vitro* culture of isolated, immature dog follicles was influenced by the stage of the follicle and gonadotropin (and dose) used. Even by studying a rather modest number of follicles from a diverse population of donors, it was possible to stimulate physical, measurable growth of both preantral and early antral follicles, but with more success in the latter. FSH was important, and it was possible to quantify steroidogenesis in small cohorts of these premature follicles, thereby indicating active theca cells for up to 3 wk of incubation. However, it was not possible to reach a growth state consistent with recovering an oocyte with a chance of maturing and fertilizing *in vitro*. These and other observations, especially lack of antrum expansion and oocyte disruptions, provided useful insight into the sensitivity of the isolated dog

follicle/oocyte complex to an *in vitro* microenvironment and, as importantly, guidance for next steps to optimize culture conditions. From a functional perspective, the priority is to promote and maintain communications between the oocyte and cumulus cells as growth proceeds in culture. Our collective observations here indicate the need to both ensure that the most appropriate substrates are available and that the follicle itself has the capacity to expand. We are encouraged by our early observations [71] that presence of growth factors (e.g., activin) may assist in meeting this first mandate. The second priority of allowing antrum enlargement might be met by borrowing from novel concepts of others [89] to create a semi-degradable, encapsulating environment that permits self-digestion from endogenously-released proteases to more closely mimic what occurs naturally in the ovary.

Declaration of interest

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

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CHAPTER THREE

Activin Promotes Growth and Antral Cavity Expansion in Domestic Dog Early Antral and Antral-Stage Ovarian Follicles

Abstract

Activin has been demonstrated to sustain cumulus-oocyte communication that in turn promotes *in vitro* follicle and oocyte growth in the mouse and cow. Here, we investigated the role of activin on survival, growth, antrum development, ability to sustain trans-zonal projections (TZPs), oocyte quality, and FSH receptor expression during *in vitro* culture of preantral (150-≤ 230 µm diameter), early antral (231-≤ 330 µm) and antral (>330 µm) domestic dog follicles encapsulated in a fibrin-alginate hydrogel with 0, 100 or 200 ng/ml rhActivin plus 0, 0.1, 1 or 10 µg/ml FSH for 12 or 21 days. Neither hormone supplementation, follicle stage, nor their interactions affected survival. All size/stage follicle groups grew ($P < 0.05$) over the culture period, although activin improved growth ($P<0.05$) and expanded antral cavity formation primarily in early antral and antral cohorts. Activin did not influence ($P > 0.05$) TZP density or *FSHR* expression after 12 days of culture; however, 100 ng/ml activin supported chromatin integrity and the maintenance of TZP density at 21 days. In summary, these results indicate that activin acts synergistically with FSH to promote growth and antral cavity expansion of the dog follicle *in vitro.* These findings serve an important foundation for developing culture microenvironments to enable rescuing the maternal genome from follicles of females that are genetically underrepresented in populations or that die before reproducing.

Introduction

Understanding of the regulators of antrum formation and oocyte development during folliculogenesis remains limited for many large mammals, including the domestic dog. Dogs experience variably long periods of anestrus that is not well understood, wherein little to no follicle development occurs [28]. Further, the dog's all-encompassing ovarian bursa [84] has limited our ability to understand *in vivo* follicle dynamics, as only antral follicles can be visualized via ultrasound once they become large enough to protrude from the surface of the ovary. These characteristics have hindered efforts to understand the drivers of folliculogenesis in the dog.

However, use of follicle culture provides a means to begin to overcome these limitations, as effects specific growth factors and their interactions may be observed over time and developmental stages *in vitro*. This knowledge would be beneficial in the advancement of contraceptives for domestic dogs, as well as our ability to rescue the maternal genome of rare/endangered canids via *in vitro* ovarian follicle culture.

Previous work by this laboratory has evaluated the effects of hormonal and physical microenvironment of the growth and development of alginate-encapsulated domestic dog ovarian follicles *in vitro* [195]. FSH, but not necessarily LH, significantly improved growth of pre- and early-antral follicles in the 3-dimensional (3D) alginate culture system. However, observations of the follicles in this study revealed that although oocytes increased in size, they became increasingly pale over the 20 d culture period which indicative of lipid loss. Furthermore, extrusion of oocytes from the follicle wall, as has been described in follicle culture from other species [95, 193, 194], was also noted.

The lipid-depleted oocytes and their extrusion from the follicle walls suggested a loss in communications between the oocyte and the granulosa cells. Trans-zonal projections (TZPs), are extensions of cumulus cells through the zona pellucida that form junctions with the oocyte. When oocytes were extruded from the follicle in our study and others [95], cumulus cells were not present, as further evidence of a breakdown of TZPs in these follicles. The protein hormone activin has been shown to maintain TZPs in cultured follicles in bovine follicle culture [196]. In the rat and human, addition of the protein hormone activin to *in vitro* follicle culture media promoted antrum development [104, 105] as well as follicle growth [103], survival [106], and oocyte maturation [102, 107]. This hormone has been shown to localize to the granulosa cells of follicles in the cow [197], pig [100], and monkey [101], and operates through a Smad signal transduction pathway to influence gene expression [108-110]. Specifically, studies in the rat have identified its role in increasing granulosa cell sensitivity to follicle stimulating hormone by increasing the numbers of FSH receptors (FSHR) on these cells [111, 112].

The objective of this study was to determine the stage-specific effects of the protein hormone activin on growth, survival and antrum formation as well as resident oocyte health within domestic dog follicles cultured in 3-dimensional system utilizing, a dynamic Fibrin-Alginate Interpenetrating Network (FA-IPN), [89]. In this system, the fibrin component of the hydrogel can be degraded away by proteases produced by the follicle as it grows, thereby softening the gel adjacent to the follicle and allowing for further growth. We hypothesized that activin would 1) act synergistically with FSH via upregulation of FSH receptors to promote follicle growth, and 2) promote antrum formation and oocyte health via maintenance of TZPs.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and media from Irvine Scientific (Irvine, CA) unless otherwise stated.

Follicle Isolation, Encapsulation and Culture

Ovaries from 28 dogs of various breeds (age 6 months to 9 years) in 20 independent culture trials were obtained during routine ovariohysterectomy at local veterinary clinics, stored on ice in L15 media with 30 µg/ml penicillin G sodium and streptomycin sulfate and 50 mM ascorbic acid, and transported to the laboratory. Although stage of reproductive cycle was not available for most individuals, the bitches were primarily in anestrus or diestrus, based on the presence of corpora lutea and absence of large antral follicles on the ovaries [81]. Pre- (100 - ≤230 µm diameter), early antral (231 - ≤330 µm), and antral follicles (331 - 500 µm) were mechanically isolated using a scalpel blade and a 25G needle within 3 h of surgery. Only follicles having at least two layers of granulosa cells with a homogeneously dark, centralized, circular oocyte and intact basement membrane were included in the study.

Isolated follicles (n = 707) were individually encapsulated in a bead of 1:1 Fibrinogen-Alginate for final concentrations of 22.5 mg/ml fibrinogen (from bovine plasma, Sigma) with 0.38% (w/v) alginate (FMC BioPolymers, Philadelphia, PA), using methods previously described [89].

Briefly, follicles in fibrinogen-alginate (5 µl) were pipetted into 50 U/ml thrombin in Tris BuPH with 40 mM $CaCl_2$. Alginate crosslinks in the presence of a divalent cation, like calcium [157], and fibrinogen polymerizes into fibrin when thrombin proteolyzes fibrinogen and activates factor XIIIa present in the fibrinogen plasma preparation [89]. Beads were then washed in *Growth Media* [88] (α-minimum essential medium, 3 mg/mL BSA, 4.2 µg/ml insulin, 3.8 µg/ml transferrin and 5 ng/ml selenium. Encapsulated follicles were then transferred to individual wells of a flat-bottomed 96-well culture plate in 100 µl *Growth Media* supplemented with specific concentrations of FSH and activin (see below). Follicles were randomly assigned to one of 12 hormone treatment groups of 0, 0.1, 1, or 10 µg/ml FSH and 0, 100 or 200 ng/ml rhActivin (4x3 factorial design; 24-55 follicles/group) and cultured at 38.5°C in humidified atmosphere of 5% CO2 for 12 or 21 days. Every 72 h, half the medium in each culture well was exchanged with fresh. On days 3, 6, 9, 12, 15, and 21 follicle diameter, survival, and antrum formation was evaluated using an inverted microscope (Leitz DM-IL, Research Instrument Limited, Falmouth, Cornwall, UK) with a heated stage and an optical micrometer. Each follicle was sized from the outer layer of the cells, and the measurements included the widest diameter and the perpendicular width to the initial assessment. The mean of these two metrics was calculated and reported as 'follicle diameter' [195]. At the onset of culture, two diameter measurements were taken – the first to the edge of the follicle basement membrane, for evaluation of initial developmental stage (preantral, early antral, or antral), and the second to the edge of the follicle plus any residual somatic cells attached after the mechanical isolation process, for evaluation of Day 0 diameter. A follicle was considered degenerate if it decreased significantly in diameter over several culture days, contained a degenerate oocyte, or if its granulosa was fragmented [87]. Follicles which did not survive to the third day of culture were considered to have had an unseen defect prior to or as result of the encapsulation process and not included in analyses, resulting in 621 follicles being included (preantral n = 145, early antral n = 359, and antral n = 117).

Antral cavity expansion, rather than development, was assessed for two reasons. First, because a portion of the follicles cultured in this study began at the early antral and antral stages of development, and therefore cavitation had already been initiated. Second, our previous study in the culture of domestic dog follicles had observed collapse of antral cavities [195], prompting our interest in not only the development, but also the maintenance and continued expansion of antral cavities over time. Therefore, 'expanded antral' follicles were characterized by the development of a large, fluid-filled cavity that was maintained over subsequent observations.

At the end of culture (Day 12 or 21), cumulus oocyte complexes (n = 213) were isolated using two 25G needles to gently tease them out of the granulosa cells. COCs were fixed in 4% paraformaldehyde for 12 hours, then stored in wash buffer (containing 0.2% sodium azide, 2% normal goat serum, 1% bovine serum albumin, 0.1 M glycine, and 0.1% Triton X-100 [198]) at 4ºC for TZP analysis. Granulosa cells from follicles cultured for 12 days in each treatment group were combined, then stored in RNAlater (Qiagen) at -80ºC for later RT-PCR.

Follicle Analysis

At the end of culture (Day 21), follicles in FA-IPN beads were fixed with Bouin's solution then embedded in paraffin blocks based on treatment group. For each block, 6 µm thick sections were cut and one slide was stained with Haemotoxylin and Eosin (H+E) to evaluate follicle morphology (intact oocyte and basement membrane, theca cells, presence of antral cavity).

TZP analysis

Actin staining on fixed COCs was achieved using Alexa Fluor 488 Rhodamine Phalloidin [198] (Invitrogen at 1:100 dilution in wash buffer) for at least 30 min at room temperature, with simultaneous nuclear stain with 1 µg/ml Hoechst 33342. Oocytes were mounted on a slide with GVA Aqueous Mounting Solution (Genemed Biotechnologies, San Francisco, CA) for imaging at 100x with an LSM 510 laser scanning confocal microscope (Carl Zeiss, Germany) with a 488 nm krypton/argon laser and BP: 420-480. TZP density was analyzed using ImageJ software (NIH,

Bethesda, MD) by taking linescans of fluorescence intensity through the zona. Intact nuclear status and oocyte size was also recorded.

Quantitative RT-PCR

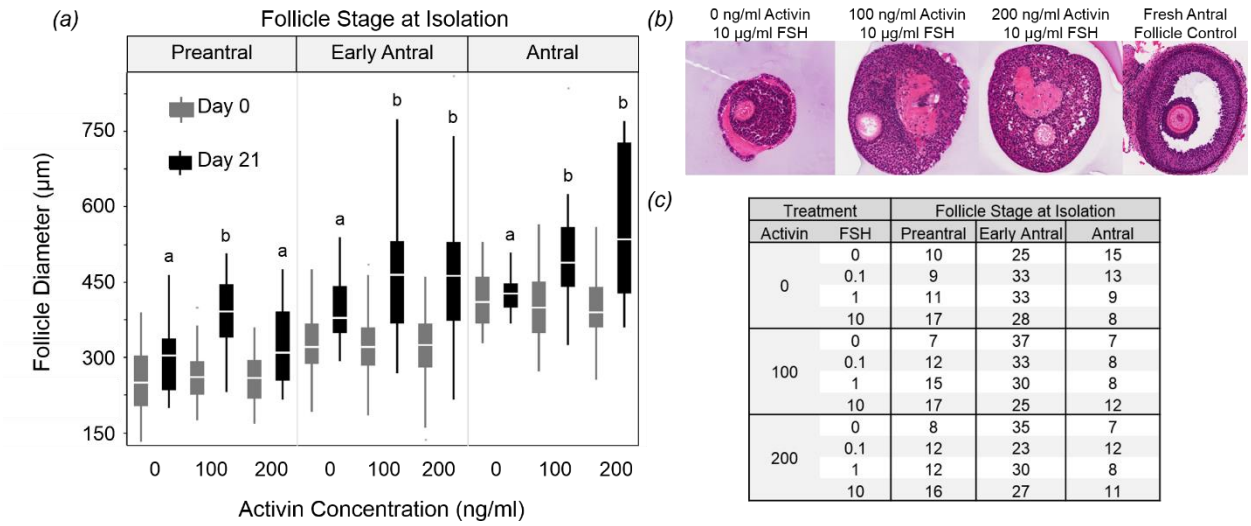
To obtain enough RNA for analysis, stored granulosa cells from all cultures were combined by treatment into two groups, resulting in two pooled samples per treatment group. RNA was extracted using Tri Reagent (Sigma), according to manufacturer's instructions, and converted to cDNA using High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). FSHR expression was assessed by SYBR RT-PCR using *GUSB* (103 bp) as the internal reference gene [199] and *FSHR* (122 bp) Forward: ATTAGCATCCTGGCCATCAC, Reverse: CCAATGCAGAGATCAGCAAA. Products were submitted for sequencing to the Cornell Institute of Biotechnology Genomics Facility to confirm match to predicted sequence for domestic dog FSH receptor.

Statistical Analysis

Results are expressed as the mean $\pm$ SEM and all analyses were completed using JMP 9.0 software (SAS, Cary, NC). Growth data (percent diameter increased from Day 0 size) was subjected to log transformation prior to use of mixed-effect ANOVA model and post-hoc Tukey-Kramer HSD test for significance. Survival differences between follicle stages, treatment groups and their interactions were evaluated using proportional hazards model. Percent antrum formation between treatment groups was compared via Chi square likelihood ratio test. Average number of TZPs per µm line of zona, proportion of intact chromatin, and oocyte growth were evaluated using nonparametric Wilcoxon test for differences between treatment groups. RT-PCR data was evaluated using the $2^{-\Delta C_T}$ method [200] followed by Wilcoxon test. Significance was set for all analyses at $P < 0.05$.

Results

All factors (FSH, Activin, the interaction between FSH and Activin, and Follicle stage at isolation) contributed significantly to follicle growth ($P < 0.05$). On average, all follicle stages experienced a significant increase in diameter over the culture period, regardless of activin treatment group ($P < 0.05$), with the exception of antral stage follicles in the absence of activin (Fig. 3.1a). Histological evaluation of follicles cultured for 21 days revealed an absence of cells with theca-like morphology, but the presence of fluid-filled cavities in many large follicles which stained pink in the presence of eosin, indicating high protein content (Fig. 3.1b). In general, increasing FSH concentrations increased the rate of follicle growth over the 21 day culture period. This effect was more pronounced in early antral and antral stage follicles, but not preantral follicles, exposed to activin (Fig. 3.2). For example, in the absence of activin, early antral stage follicles cultured in 0.1 µg/ml FSH for 21 days increased in diameter 18.5 ± 3.7%, and those cultured with 1 µg/ml FSH grew 22.4 ± 5.3%. Conversely, those cultured in 100 ng/ml activin in these two FSH concentrations grew 44.0 + 6.4 and 74.2 + 15.2%, respectively. However, increasing activin concentrations from 100 to 200 ng/ml rarely provided additional benefit to follicle growth, with the exception of antral follicles. Antral follicles, in addition to responding very poorly in the absence of FSH, also required activin in order to grow when cultured in high concentrations of FSH (10 □g/ml) ($P < 0.05$).



Treatment		Follicle Stage at Isolation		
Activin	FSH	Preantral	Early Antral	Antral
0	0	10	25	15
	0.1	9	33	13
	1	11	33	9
	10	17	28	8
100	0	7	37	7
	0.1	12	33	8
	1	15	30	8
	10	17	25	12
200	0	8	35	7
	0.1	12	23	12
	1	12	30	8
	10	16	27	11

Figure 3.1: *(a)* change in follicle diameters over culture period based on stage at initial isolation and activin treatment group (0, 100 and 200 ng/ml rhActivin), letters indicate significant differences between activin treatment groups within day and follicle stage at isolation, *(b)* Histology via H+E stain of follicles cultured 21 days in 10 µg/ml FSH and 0, 100 or 200 ng/ml activin, and *(c)* numbers of follicles cultured in each treatment group.

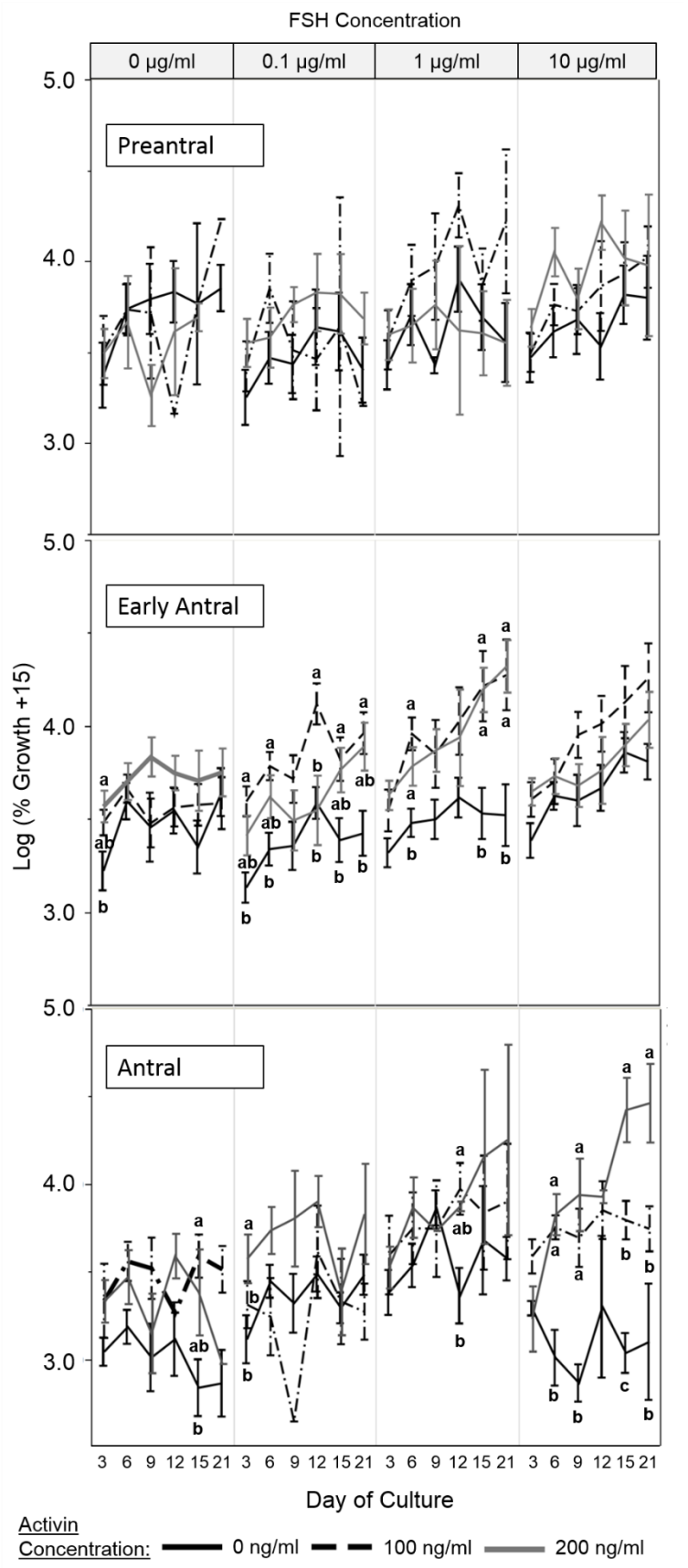

FSH Concentration
0 µg/ml
0.1 µg/ml
1 µg/ml
10 µg/ml
Preantral
Early Antral
Antral
Log (% Growth +15)
5.0
4.0
3.0
3 6 9 12 15 21
Day of Culture
Activin Concentration:
0 ng/ml
100 ng/ml
200 ng/ml

Figure 3.2: Follicle growth (log transformation of % diameter increase compared to Day 0 size) of preantral, early antral, and antral stage follicles cultured for 21 days in various concentrations of FSH (0, 0.1, 1 and 10 □g/ml) and activin (0, 100 and 200 ng/ml). Letters indicate significant differences between activin treatment groups within the same culture day, FSH concentration and developmental stage.

No significant effect of follicle stage, activin or FSH concentration, nor their interaction was observed on survival ($P > 0.05$), although follicle loss due to oocyte extrusion was noted (Fig. 3.3). Preantral stage follicles cultured with FSH in the absence of activin supplementation, and antral follicles in the presence of high concentrations of both hormones appeared particularly susceptible to this phenomenon. Overall, 14.0, 8.6, and 6.2% of preantral, early antral, and antral stage follicles, respectively, experienced oocyte extrusion in culture and the majority of these events (73.5%) took place during the first 6 days *in vitro*.

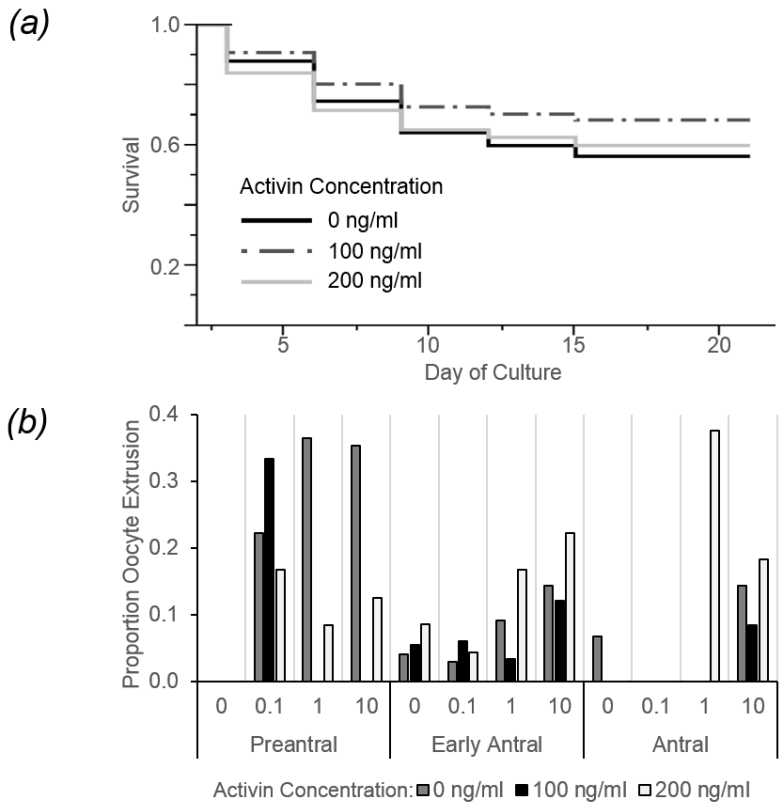


Figure 3.3: Follicle survival *(a)* Survival of follicles cultured in various concentrations of activin during 21 day culture period, and *(b)* proportion of oocyte extrusion based on original stage at isolation (preantral, early antral or antral) and hormone treatment group (0, 100 and 200 ng/ml rhActivin and 0, 0.1, 1 and 10 µg/ml FSH)

Antral cavity expansion was also observed in follicles over the course of the culture period (Fig. 3.4a). Interestingly, no follicles which began as “preantral” at the onset of culture developed to this expanded antral cavity. Regardless, follicles which did develop these large cavities did so exclusively in the presence of activin, and with increasing frequency in the dual presence of FSH (Fig. 3.4b). Of the 36 follicles (5.0% of total follicles cultured) which reached this advanced stage, the majority (61.1%) did so between days 10-12 of culture.

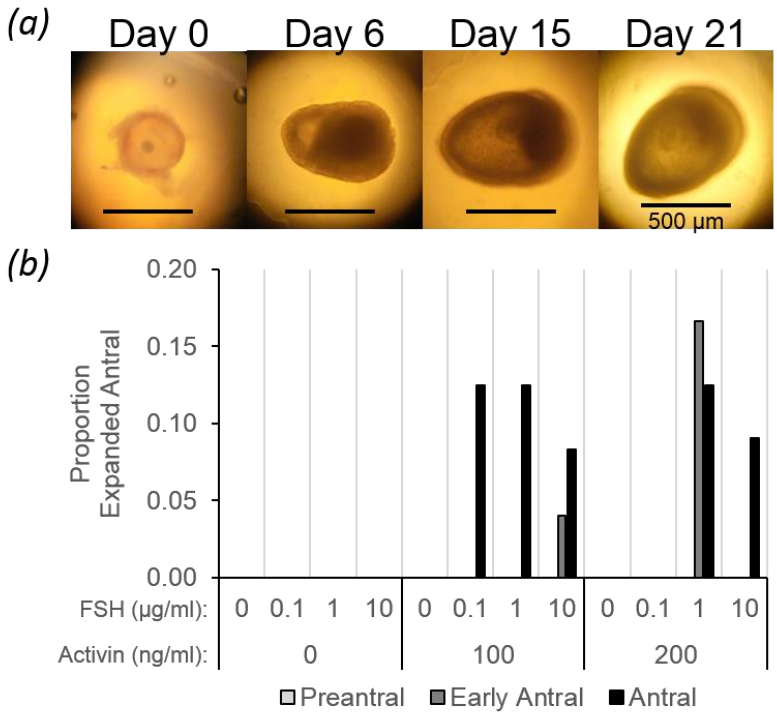


Figure 3.4: Antral cavity formation *(a)* Photomicrographs of a single early antral follicle cultured in 100 ng/ml activin and 1 µg/ml FSH developing an antral cavity over 21 days culture, *(b)* Proportion of follicles developing expanded antral cavities based on original stage at isolation and hormone treatment group (0, 100 and 200 ng/ml rhActivin and 0, 0.1, 1 and 10 µg/ml FSH).

Maintenance of trans-zonal projections was evaluated at the two culture time points (day 12 or 21). Very few TZPs were maintained in follicles treated with high concentrations of FSH, regardless of the culture duration (Fig. 3.5a). There was no significant effect of activin supplementation on TZP density in the first 12 days of culture (average 0.06 ± 0.01 in the presence of activin, and 0.08 ± 0.01 TZPs/µm zona without), but follicles cultured for 21 days displayed a significant reduction in TZPs in the absence of activin. Increased activin concentration (200 ng/ml versus 100 ng/ml) provided no additional benefit to TZP maintenance, and actually displayed a significant reduction in TZP density in 0 and 0.1 ug/ml FSH compared to those in 100 ng/ml activin on day 12. However, even in the presence of activin there was a

reduction in TZP density compared to that of freshly isolated COCs (average 0.32 ± 0.01 TZPs/µm, compared to an overall average of 0.06 ± 0.01 TZPs/µm for cultured follicles). Vesiculation was also observed along the cortical edge of some oocytes at the junction with TZPs (Fig. 3.5b).

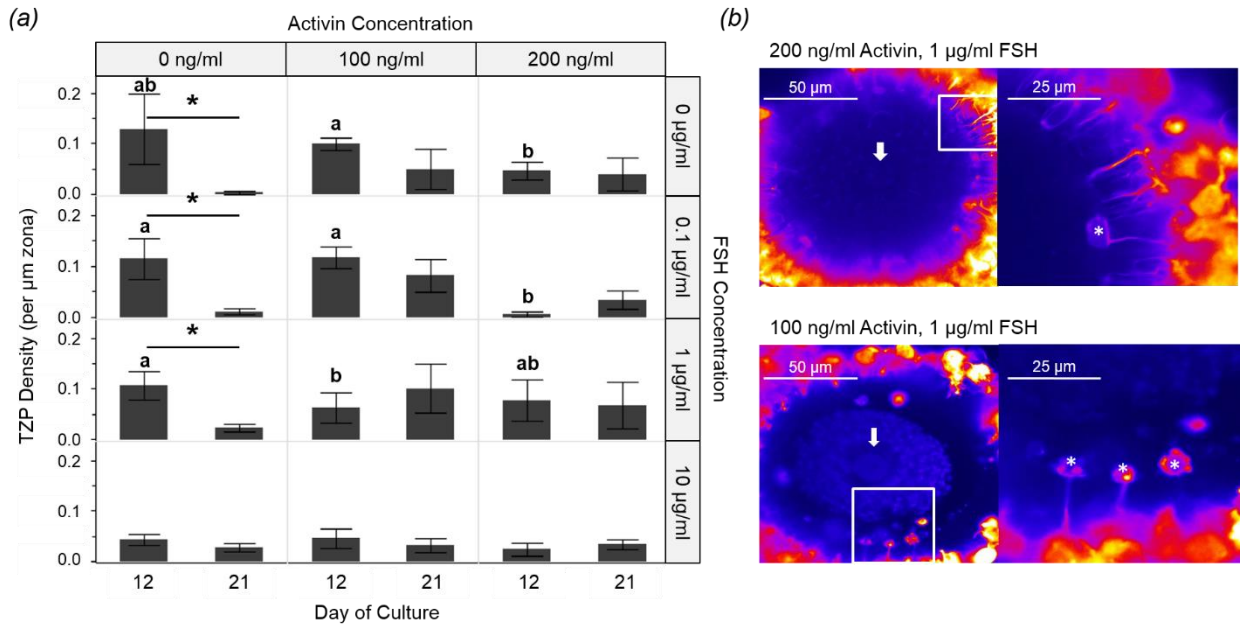


Figure 3.5: TZPs in oocytes from cultured follicles. *(a)* TZP density per treatment group (0, 100 and 200 ng/ml rhActivin and 0, 0.1, 1 and 10 µg/ml FSH) for follicles cultured 12 or 21 days. Asterisks (*) indicate differences between days within treatment, and letters mark significant differences between treatments on each day. *(b)* confocal microscopy images of TZPs, with arrow marking nucleus and asterisks (*) marking vesiculation. Second images are magnifications of white boxed areas.

Oocytes grew significantly after 12 days culture in all treatment groups, except for in the absence of hormone supplementation ($P < 0.05$, Fig. 3.6a). Oocyte growth either plateaued between day 12 and 21, or oocyte size actually reduced during the second half of culture. After 12 days of culture, presence of activin significantly ($P < 0.05$) improved proportion of intact chromatin (Fig. 3.6b). Additionally, no significant differences were observed in FSH receptor

expression among activin treatment groups (Fig. 3.7), although high variation among replicates was observed, particularly at the 200 ng/ml activin concentration.

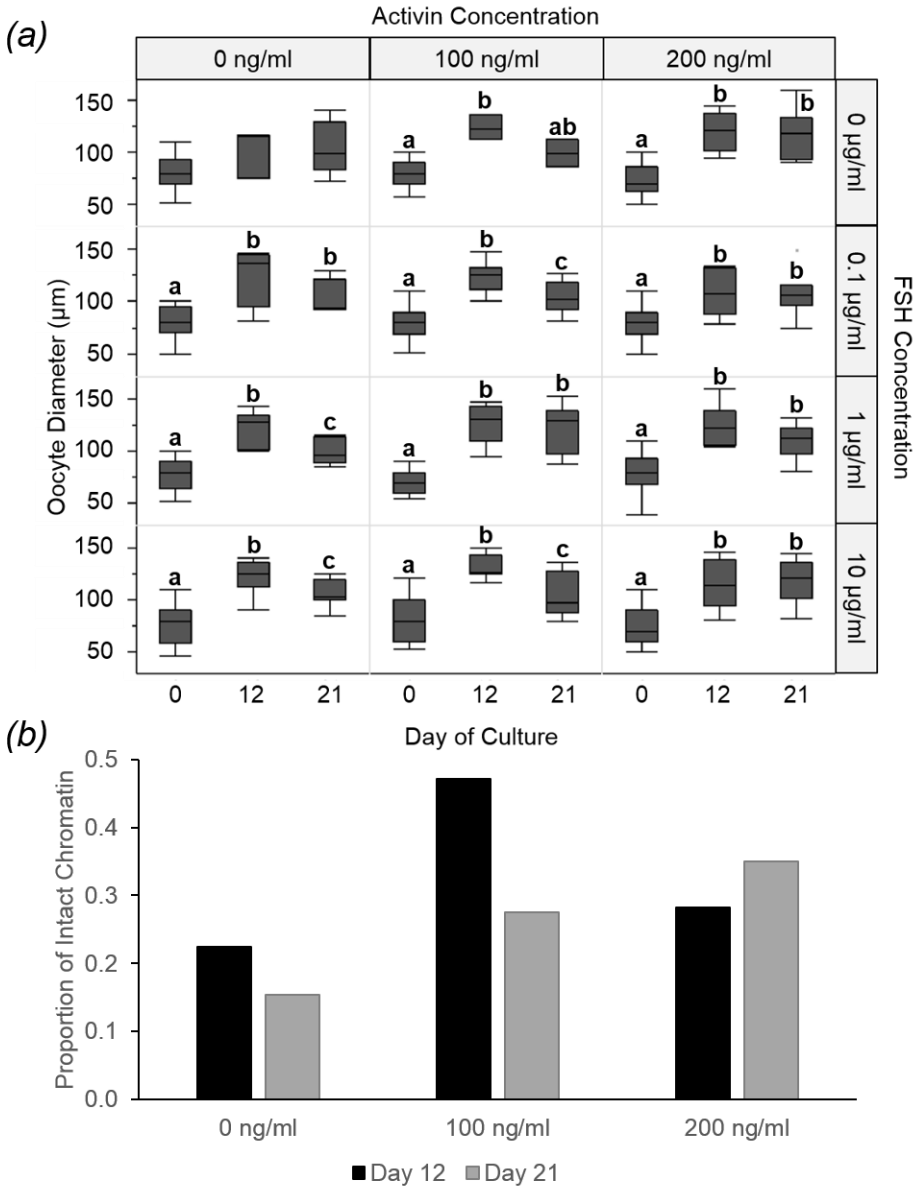


Figure 3.6: Health of oocytes from cultured follicles *(a)* Oocyte growth, with letters indicating significant differences between oocyte sizes between days for each treatment (0, 100 and 200 ng/ml rhActivin and 0, 0.1, 1 and 10 µg/ml FSH) and *(b)* proportion of intact chromatin by activin treatment group for follicles cultured 12 or 21 days

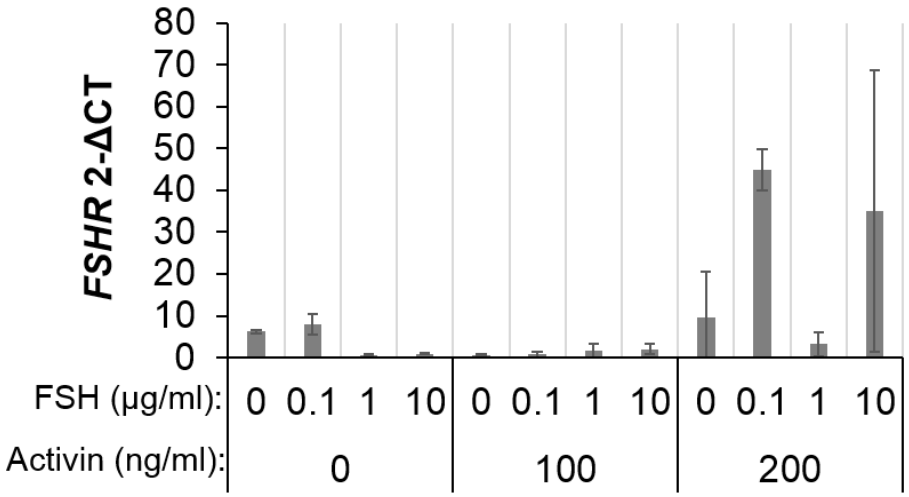


Figure 3.7: mRNA expression of *FSHR* with *GUSB* as the internal reference gene in granulosa cells of Day 12 cultured follicles from 6 independent cultures, combined by treatment group (0, 100 and 200 ng/ml rhActivin and 0, 0.1, 1 and 10 µg/ml FSH) into two sets of pooled samples.

Discussion

The protein hormone activin has been shown to improve follicle growth [103], oocyte maturation [102, 107], maintenance of TZPs [196], FSH sensitivity [111, 112], and antrum formation rates [104, 105] in the cow, rat, human, mouse, and zebrafish. Our objective was to investigate the effect of activin supplementation on domestic dog follicle growth, survival, antral cavity development and oocyte health in a dynamic, 3D *in vitro* culture system. This study made four main discoveries. First, activin acted synergistically with FSH to promote follicle growth and antrum development in early antral and antral stage follicles. Second, activin supplementation promoted the maintenance of TZPs between days 12 and 21 of culture. Third, there was little to no effect of activin supplementation on oocyte growth but activin improved proportions of oocytes with intact nuclei in short term (12 d) culture. Finally, there was no significant effect of activin supplementation on FSHR expression as evaluated here.

In the present study, we demonstrated stage-specific effects of activin on growth of dog follicles. Specifically, activin improved growth in the presence of low levels of FSH in early antral

follicles. Antral stage follicles in the presence of high FSH concentrations required activin for growth. However, when controlling for effect of FSH, no statistically significant effect of activin on growth was observed in preantral follicles. In other species, including the nonhuman primate, human, and goat, activin is thought to be produced by preantral through antral stage follicles [99, 101, 201], and acts in a paracrine manner to promote FSH receptor expression, as well as oocyte and follicle growth. Although activin has been shown to promote growth [196] in preantral follicles in some species, our growth data suggests this may not be the case, or at minimum does not occur to the same extent, for domestic dog preantral follicles.

Murine studies have described an interesting age-specific effect of activin that may explain the lack of observable difference of activin supplementation on preantral follicle development [202]. In that study, preantral follicles from immature mice responded with increased growth in the presence of activin, whereas preantral follicles from adult mice did not stimulate follicle growth. All dog ovaries included in this study were from pubertal (> 6 mo) or adult animals, so it is uncertain if the lack of preantral follicle responsiveness in the current study was related to age. If so it suggests an interesting avenue of future study on the influence of past exposure to the hormones involved in reproductive cyclicity on current responsiveness of follicle cohorts. In bovine studies, supplementation of activin A to cultured granulosa cells from antral and preovulatory stage follicles resulted in a delay of luteinization and subsequent progesterone production [203], which was resumed after addition of follistatin (activin binding protein) to culture [204]. This suggests activin may act as a survival factor for antral stage follicles by preventing premature luteinization. Luteinization has been observed in granulosa cells of activin-deficient [205] and Smad4 KO mice [206]. Marmoset monkey granulosa cells from antral follicles displayed a reduced progesterone-secretion response to LH in the presence of activin [207]. Although no significant influence of activin on antral stage follicles was noted based on our survival evaluations in our statistical model, the lack of significant growth of antral follicles in the absence of activin may indicate this is the case for the dog as well.

Extrusion of oocytes is not uncommon, and has been noted in the culture of mouse [194], human [95], goat [193], and domestic dog [195] ovarian follicle culture. It has been hypothesized to be the result of a loss of communication between the oocyte and surrounding somatic cells, as extruded oocytes are generally voided of cumulus cells. In the current study, extrusion appeared to be more common in preantral stage follicles compared to follicles in later stages of development, and tended to occur within the first few days of culture. It is likely that these small follicles are more susceptible to insult to their basement membranes via mechanical isolation. Data from goat follicle culture have shown significant reduction in oocyte extrusion rates when FSH was supplemented in incrementally increasing concentrations over the culture period, compared to steady-state FSH controls [193]. In that study, it was suggested that increasing FSH concentrations were required to promote continued follicle survival as culture progressed and follicles developed to the early antral, FSH-dependent stage. Incrementally increasing FSH concentrations have been previously utilized in the culture of dog oocytes, with promising results regarding growth and antrum formation [93], therefore future studies should evaluate the dual effect of incrementally increasing FSH and activin supplementation of oocyte extrusion in long term follicle culture.

We were encouraged by our observation that antral cavities were able to be maintained and continued to expand in this culture system. In our previous investigation, development of large antral cavities was infrequent and not sustained during long-term culture. We speculated that the rigidity of the alginate hydrogel may have limited follicle growth observed in the earlier study [195], which was our rationale for utilizing the more permissive FA-IPN here. In the current study, several follicles developed expanded antral cavities and reached sizes > 800 µm diameter, a significant improvement to the ~ 500 µm diameter growth cap we observed in our previous investigations. It has been shown that oocytes from dog antral follicles > 2 mm diameter have much higher rates of meiotic resumption compared to smaller follicle counterparts *in vitro* [132]. The growth and expansion of large follicles in this study, therefore,

represent another step toward that goal. It is also notable that studies culturing dog follicles in a two-dimensional (2D) culture system have reported much higher rates of antral cavity development (up to 100% of follicles cultured for 18 days) [93, 208], although direct comparisons are not possible due to different definitions of follicle stages at isolation and designations of antral cavity development. Further investigations are necessary to evaluate if the FA-IPN system is beneficial in the maintenance of the 3D structure of larger follicles (in the millimeter diameter range) compared to 2D systems, or if it, too, becomes restrictive to growth due to the alginate component at these sizes.

Interestingly, histological evaluations of follicles cultured in our system revealed strong eosin staining in the follicular fluid of these antral follicles. Eosin specifically basic, or positively charged molecules and proteins. The mechanisms of antral cavity development are not fully elucidated, although hypotheses include osmotic gradient due to the secretion of large proteins into the cavity, as well as an increase in aquaporin expression in granulosa cells to facilitate the movement of water into the follicle [65]. The large molecules believed to contribute to this osmotic gradient have been suggested to be negatively charged molecules, such as proteoglycans [66], but further exploration is still needed in this area. If large proteins are the source of the staining in our study, it may suggest that fluid transport was not supported in the culture system, indicating an issue with aquaporins, as the primary mediators of water permeability in rat antral follicles [65]. Aquaporins 1,3, and 4 have been shown to express at levels several fold higher in theca cells compared to granulosa cells of human preovulatory follicles [209], thus this is likely related to the absence of theca cells observed in follicles cultured long-term. This absence was curious, as mechanical dissection allowed for the presence of associated theca and stromal cells surrounding isolated follicles at the initiation of culture. Further, although activin promoted expansion of existing or early antral cavities in the current study, initiation of antral cavities in preantral stage follicles did not occur. This, too, may be related to the lack of theca cell presence in the culture system. Theca cell growth and

development has been shown to be promoted by kit-ligand in bovine follicle cell culture [210], suggesting this growth factor may be required in single-follicle *in vitro* culture systems to support theca cell layer development and, thereby, water permeability.

Contrary to our hypothesis, supplementation of activin did not have a significant effect on the density of trans-zonal projections over the first half of culture (day 12). Although an effect was noted during the second half of culture, our observations regarding oocyte size, TZP density and intact chromatin status on Day 21 suggest that many follicles did not continue to develop beyond approximately 12 days of culture. This may have been a product of small sample size and high variation among COCs. We could also speculate that activin did promote TZP maintenance in the first 12 days of culture as well, but differences did not become apparent between treatment groups until later in culture because establishment of new TZPs did not occur as granulosa/cumulus cells continued to proliferate. Understanding of the establishment of TZPs is only recently being explored in other species. Mora et al have demonstrated that preantral murine follicles rely on calcium-dependent cadherins in the oocyte for TZP contact between the oocyte and surrounding somatic cells [211]. Recent studies in mice have also demonstrated a role for focal adhesion kinase (PTK2), a factor involved in cytoskeleton regulation and adherens junction assembly [212]. The cause of the observed vesiculation at the oocyte cortex at the junctions of some TZPs is not known, but a similar phenomenon has previously been described by Viaris de Lesegno et al in the ultrastructural evaluation of *in vitro* matured canine oocytes [213]. In that study, deeply embedded TZPs with a ‘bulbous superficial ending’ were noted, and it was hypothesized they may have been the result of folding of the oocyte plasma membrane onto itself. Regardless, improved understanding of factors involved in the establishment and maintenance of TZPs will be necessary to extend our ability to maintain healthy oocytes in long-term (> 12 day) follicle culture.

Dog oocytes reach their maximal size (approximately 113 µm, range 86.2 to 131.5 [77]) around the early antral stage [94], therefore we did not anticipate additional growth once these

diameters were reached by Day 12 of culture in the current study. However, oocytes evaluated on Day 21 were generally smaller than those collected on Day 12. Combined with data on intact chromatin status, it is evident that oocyte survival beyond Day 12 of culture was reduced. This presents a challenge for identifying healthy oocytes in *in vitro* follicle culture, as the follicles from which these oocytes were collected appeared live, morphologically normal, and in many cases displayed continued growth (likely due to granulosa cell proliferation in the presence of the hormone supplementation). Dyes like Neutral red have previously been utilized to assess viability without compromising follicle integrity in culture [214] and may be a useful tool to identify viability over the course of long-term culture as well. Improved insight into the timing of oocyte loss within cultured follicles will be important for understanding what factors might be involved in supporting survival at each stage of folliculogenesis.

No consistent effect of activin supplementation on FSH receptor expression was observed in the current study. Based on the growth data, there was a dramatic stage-specific effect of activin on growth that acted synergistically with FSH, except in preantral follicles. Although preantral stage follicles have been shown to have increased FSHR expression and resulting growth in the presence of activin in the mouse [215], this is not likely the case in the domestic dog. Follicles cultured in this study were combined based on activin treatment group in order to obtain enough RNA for analysis, but this may have obscured the effect of activin on FSHR as follicles in different stages of development were grouped. While follicles in 0.1 µg/ml FSH plus 200 ng/ml activin had significantly higher FSHR expression compared with the other treatment groups, the variability between the two sets of pooled samples overall makes it difficult to draw a strong conclusion from this observation. Moreover, it has been shown that long term exposure to FSH in the presence of activin results in the down-regulation of FSHR [215, 216], which explain the lack of beneficial effect of activin on gonadotropin binding site in Day 12 follicles.

In summary, we have demonstrated a significant effect of activin on early antral and antral stage dog follicle growth and antrum expansion *in vitro*. As large antral follicle development is critically required for the development of a meiotically competent oocyte in the domestic dog, this represents an important step for *in vitro* folliculogenesis in this and other canid species. Oocytes cultured in this system for 12 days demonstrated growth and improved proportion of intact chromatin in the presence of activin, which holds promise for our ability to collect healthy oocytes from domestic dog *in vitro* follicle culture. Future work should focus on the mechanisms of antral cavity initiation and TZP junction establishment in dog follicles.

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CHAPTER FOUR

Anti-Müllerian Hormone in the Domestic Dog during the Anestrus to Estrus Transition*

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* Nagashima, J.B., Hansen, B.S., Songsasen, N., Travis, A.J, Place, N.J. Anti-Müllerian Hormone in the Domestic Dog during the Anestrus to Estrus Transition. *Manuscript in submission*

Abstract

The reproductive cycle of the domestic dog features a long period of relative ovarian inactivity, or anestrus. The mechanism of anestrus termination/estrus resumption is not yet fully understood, which presents a challenge to the development of estrus induction protocols. In this study we assess the possibility that anti-Müllerian hormone (AMH) might play a role in this transition by characterizing its patterns of expression in the circulation during the transition from anestrus to estrus and in all stages of ovarian follicular growth. Serum samples from five beagles (2.0-4.5 yr) were collected 3 times per week at least 30 d prior to the onset of estrus and assessed for AMH concentrations. Serum AMH concentration increased significantly during the transition from anestrus to proestrus, and then declined back to the anestrus baseline beginning on day -4 before the LH surge, which was determined by changes in serum progesterone concentrations. Cortical sections of ovaries from females undergoing routine ovariohysterectomy (aged 8 mo - 5 yr, n = 4) were evaluated for AMH by immunohistochemistry. Preantral and small antral follicles were most strongly immunoreactive for AMH. These data suggest that the increase in the number of antral follicles is associated with the rise in serum AMH as the anestrus period comes to an end. The rise in AMH might be useful in predicting the onset of estrus, and therefore assist with the optimization of estrus induction protocols and possibly other assisted reproductive technologies.

Introduction

Assisted reproductive technologies in dogs have met with very limited success, including relatively basic interventions such as follicle/oocyte maturation and timed ovulation. Some features of the reproductive biology of the dog account for this poor success. A typical dog reproductive cycle consists of a three-day to three-week proestrus, and an approximately one week estrus during which an LH surge occurs, followed by ovulation two days later [1]. During the subsequent two month diestrus, the corpus luteum produces high levels of progesterone

regardless of pregnancy status [2, 3]. This is followed by a prolonged and variable anestrous period, lasting 2-10 months [4]. As a result, dogs rarely experience estrus more than twice a year, often with highly variable inter-estrus intervals. Additionally, oocytes are ovulated at the germinal vesicle stage and require an additional 48-72 hours in the oviduct to resume meiosis and become fertilization-competent [5]. The mechanism of meiosis resumption in dog oocytes is not known. A marker that better predicts or may be utilized to control ovulation would improve our ability to collect oocytes at specific developmental stages from the dog. This, in turn, would aid efforts to develop assisted reproductive technologies, such as *in vitro* oocyte maturation (IVM) and *in vitro* fertilization (IVF), which have never been performed successfully in canids.

Current diagnostic tests that are meant to predict the onset of the next ovulation in the dog are of limited usefulness, particularly with regard to giving both advanced and specific notice of the LH surge. This is because the hormones evaluated to date have poor predictive value either due to 1) substantial variation in their concentrations, or 2) predictable changes in concentration occur too close to ovulation to make the necessary preparations to stimulate and harvest follicles before ovulation. For example, although serum luteinizing hormone (LH) is elevated approximately three weeks before ovulation, it is episodic and variable in its release [6, 7]. Progesterone also becomes elevated in serum due to preovulatory luteinization of the ovarian follicles; however, it only reaches ovulation-predicting levels on the day of the LH surge and therefore provides just two-days' notice of ovulation [8, 9]. Therefore, physical markers of proestrus resumption (swollen vulva, serosanguinous vaginal discharge) are still widely used to help anticipate ovulation, even though onset of these signs only indicates that estrus will likely occur sometime in the next three weeks. Because anti-Müllerian hormone (AMH) has been shown to be a useful tool in assisted reproductive technologies in women [10-12] and some production animals [13-15], we embarked on the present study to evaluate AMH for its utility in monitoring reproductive cycles in the domestic dog

Anti-Müllerian hormone, also known as Müllerian Inhibiting Substance (MIS), is a dimeric glycoprotein of the TGFβ superfamily [16, 17]. Anti-Müllerian hormone is perhaps best known for its secretion from Sertoli cells in the fetal testes which leads to the regression of the Müllerian ducts in males [18]. However, AMH is also expressed in the ovaries after Müllerian duct development is complete, and it has been shown to inhibit primordial follicle recruitment [19] and counteract the action of follicle stimulating hormone (FSH) on small antral stage follicles [20, 21]. In the ovary, AMH is localized to granulosa cells of growing follicles, with preantral and small antral stage follicles having the greatest expression in several mammalian species [17, 22-24]. The AMH signal decreases or disappears in large, preovulatory follicles [17], and is absent in corpora lutea [25]. The knowledge that AMH is produced by growing follicles has been utilized recently in human fertility research [26] and production animal breeding [13, 27] as a marker of the ovarian reserve. More primordial follicles in the ovary is correlated with increased numbers of antral stage follicles, and each have been correlated with higher circulating AMH concentrations [28, 29]. It has also been evaluated in a variety of species as a potential marker for predicting response to ovarian stimulation protocols [12, 14], as it indicates more growing follicles are present to respond to exogenous gonadotropins. Investigations have been made on the use of this hormone in predicting IVF success [30], embryo quality [15, 31], and pregnancy outcome [10, 12], with mixed results regarding the latter.

To our knowledge, the only study of AMH in the dog demonstrated that its presence or absence in blood serum distinguishes between intact and spayed bitches [32]. AMH is thought to be relatively stable across the estrus/menstrual cycle in horses and women [13, 33]. However, serum AMH concentrations are significantly higher in Siberian hamsters in long day lengths (breeding) than in short day lengths (reproductively quiescent – anestrus) [34]. Long periods of anestrus in domestic dogs are not seasonal (other than the Basenji breed [35]), but it is unknown if and how AMH values in dogs vary across the reproductive cycle or during anestrus. Using a combination of enzyme-linked immunosorbent assay (ELISA) and

immunohistochemistry (IHC), we assessed circulating AMH concentrations in dogs during the transition from anestrus to estrus and evaluated AMH expression in the ovaries. We hypothesized that AMH is produced by the granulosa cells of preantral and small antral follicles, as in other mammals, and that changes in serum AMH concentration might help to predict when the transition from anestrus to estrus will occur in individual dogs.

Materials and Methods

Experiment 1: Serum AMH Concentration

Animals

Five proven-breeder beagles (aged 2.0- 4.5 years) from Marshall BioResources (North Rose, NY) were monitored from natural (unsynchronized) late anestrus through estrus. Their study enrollment during late anestrus was based on previous inter-estrus interval durations and known date of last whelp. Animals were group housed on a 12h:12h light:dark cycle, fed laboratory canine diet #5006 once daily (LabDiet, St. Louis, MO), and provided water *ad libitum*. For estrous monitoring, blood was drawn alternately from right and left cephalic and saphenous veins 3-7 days per week, with increased frequency when proestrus was suspected, either from the presence of bloody discharge from the vulva and/or serum progesterone values higher than 0.4 ng/ml. Collected blood was allowed to clot, then centrifuged at 700*g* for 10 min to separate serum. The days of the LH surge (day 0) and ovulation (day 2) were established by progesterone values of 1.5-2.5 and 4.5-5.5 ng/ml, respectively [8, 9]. Serum was stored at -80°C until the time of AMH assay. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University.

Hormone Assays

Serum AMH concentrations were measured by AMH Gen II ELISA (Beckman Coulter, Brea, CA), which was validated for dogs. A serial dilution of canine serum was parallel to the standard curve of the assay. Samples were run in duplicate and intra- and inter- assay

coefficients of variation were 4.7 and 15.4%, respectively. Progesterone was evaluated via chemiluminescent immunoassay (Immulite, Diagnostic Product Corporation, Los Angeles, CA), previously validated for use in the domestic dog [9], with intra- and inter- assay coefficients of variation of 9.8 and 9.3%, respectively.

Statistics

Excel software (Microsoft, Redmond, WA) was used to calculate individual baseline serum AMH concentrations for each animal, according to Brown et al. (1999). Briefly, all concentrations assessed during the one-month time frame for a given individual were included in the analysis, followed by the exclusion of values that were two standard deviations above the mean by an iterative process. The average of the remaining measurements established the baseline AMH concentration for each dog.

Experiment 2: Ovarian AMH Immunohistochemistry

Ovarian Tissue Collection

Ovaries from mixed breed dogs (aged 8 mo - 5 yr) were obtained after routine ovariohysterectomy at local spay clinics. Detailed reproductive history was generally not available for these animals, however staging of reproductive cycle was estimated based on client history (recent litter, etc.), physical examination (swollen vulva, serosanguinous discharge), and distribution of follicle stages in the ovary. Strips of ovarian cortices (approx. 2 mm thick) or whole preovulatory follicles (approx. 6 mm diameter, available from one dog in proestrus) were cut from the ovary using a scalpel blade and scissors. Tissues were fixed in Bouin’s solution (Sigma, St. Louis, MO) overnight for IHC then stored in 70% ethanol before embedding in paraffin. Follicle stages were determined by morphology (preantral = more than two layers of granulosa cells surrounding a centralized oocyte; early antral = multiple layers of granulosa cells and small pockets of developing antral cavities; antral = coalesced single antral cavity, <4 mm diameter; preovulatory, ≥4 mm diameter) [36, 37].

2.2.2. *AMH Immunohistochemistry*

Immunohistochemistry for AMH in dog ovarian cortex sections was performed as previously described for hamsters [38]. Briefly, 6 µm sections were deparaffinized and antigen retrieval was performed via Vector Antigen Unmasking Solution (Vector Labs, Burlingame, CA). After blocking (10% normal rabbit serum in blocking buffer), sections were exposed to polyclonal goat anti-MIS IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, raised against C-terminus of human MIS, 1:200 dilution) for 16 hours, followed by biotinylated rabbit anti-goat IgG secondary antibody (Santa Cruz). Sections were then exposed to Vectastain Elite ABC solution (Vector Labs), NovaRed Peroxidase Substrate Solution (Vector Labs) and counterstained with hematoxylin (Thermo Scientific, Waltham, MA). A negative control was generated by omitting the primary antibody.

Results/Discussion

Currently, the ability to predict and control the reproductive cycles of domestic dogs for assisted reproductive technologies is limited, and therefore we evaluated AMH in dogs because it has proven to be a useful marker in other species, including human [10, 30]. Serum AMH concentrations were measured in a total of 91 samples from five dogs between day -37 and day 3 (relative to the LH surge) of their reproductive cycles (average 18.2 samples/dog). Although all dogs were sampled during their peak reproductive years (2.0-4.5 y), baseline AMH concentrations varied considerably among individuals (Table 4.1, Fig.4.1). In four of five dogs, serum AMH concentration increased two-fold above baseline between days -9 and -8 relative to the LH surge. In the remaining dog (#341) AMH increased to a maximum of 1.7 times baseline on day -6 relative to the LH surge. Significant changes in serum AMH concentration also have been observed over the course of the estrous cycle in the cow [39], wherein concentrations peaked on the first day of estrus (day 1), followed by a rapid decline to nadir levels by day 6. Thereafter, they gradually increased again until the next estrus. In women, some studies have suggested stable expression over the course of the menstrual cycle [33, 40], whereas other

studies have noted that women with higher baseline AMH display more variability in circulating AMH over the periovulatory period [41, 42]. The temporal rise in AMH concentration in dogs, as well as the variation seen in baseline values among individuals, emphasizes the need for special care in using AMH as a marker for various reproductive metrics in dogs and potentially other species.

Dog ID	Baseline AMH (ng/ml)	Peak AMH (ng/ml)
004	0.21	0.56
311	0.27	0.85
341	0.58	0.99
419	0.32	0.70
546	0.28	0.56

Table 4.1: Individual baseline and peak AMH concentrations for five beagles in their reproductive prime.

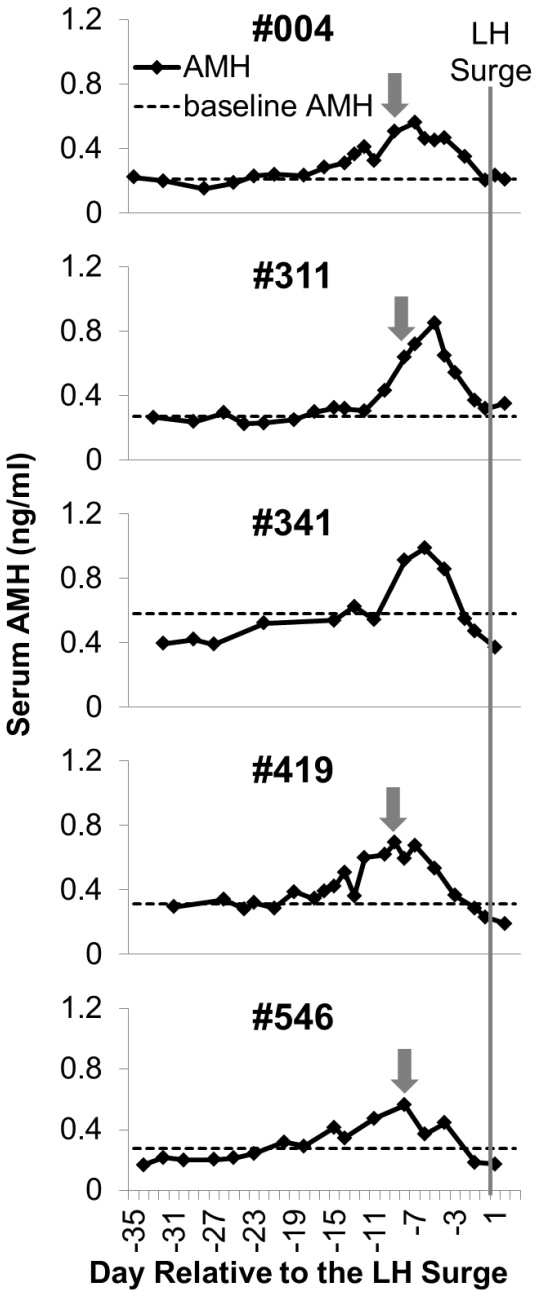

Figure 4.1: Individual serum AMH concentrations for five beagles from days -35 to 3 relative to the LH surge. Dashed lines indicate baseline AMH calculated for each individual, and the solid grey line marks the day of the LH surge (day 0). Arrows show the day when the AMH concentration first increased two-fold above baseline in four of five dogs.

In this study, mean serum AMH concentration increased from 0.30 ± 0.01 to 0.64 ± 0.03 ng/ml (Fig. 4.2A, $P < 0.05$) during proestrus, and then declined sharply back to baseline values beginning day -4 prior to the LH surge (Fig. 4.2A). Interestingly, the rise in serum AMH concentration observed in dogs in the present study also coincides with increasing numbers of antral follicles observed via ultrasound over the course of the anestrus to estrus transition [37] (Fig. 4.2B,C). The study by England et al. (2009) demonstrated a significant rise in the number of small antral (1-3 mm) follicles beginning around day -14 relative to the LH surge, peaking around day -6. At this point, presumably some follicles from this group undergo selection for ovulation, whereas others undergo atresia. Between days -6 and 0 the numbers of early antral follicles decline and the numbers of pre-ovulatory follicles increase (Fig. 4.2B,C; England et al. 2009). Serum AMH concentrations from this study appear to correspond with the rise and fall in the numbers of antral follicles. For our immunohistochemical data, immunoreactivity for AMH was minimal in domestic dog primordial follicles (Fig. 4.3A), most intense in the granulosa cells of preantral through small antral follicles (Fig. 4.3B, D), and substantially reduced in pre-ovulatory follicles (Fig. 4.3C). The negative control lacked any immunoreactivity for AMH (Fig. 4.3E). Based on these observations, we propose the rise in serum AMH during proestrus is the result of increased numbers of antral follicles producing this hormone and secreting it into the circulation (Fig. 4.2B and C). The subsequent sharp fall in AMH concentration is likely the result of this cohort of follicles simultaneously being selected to become pre-ovulatory and undergoing atresia.

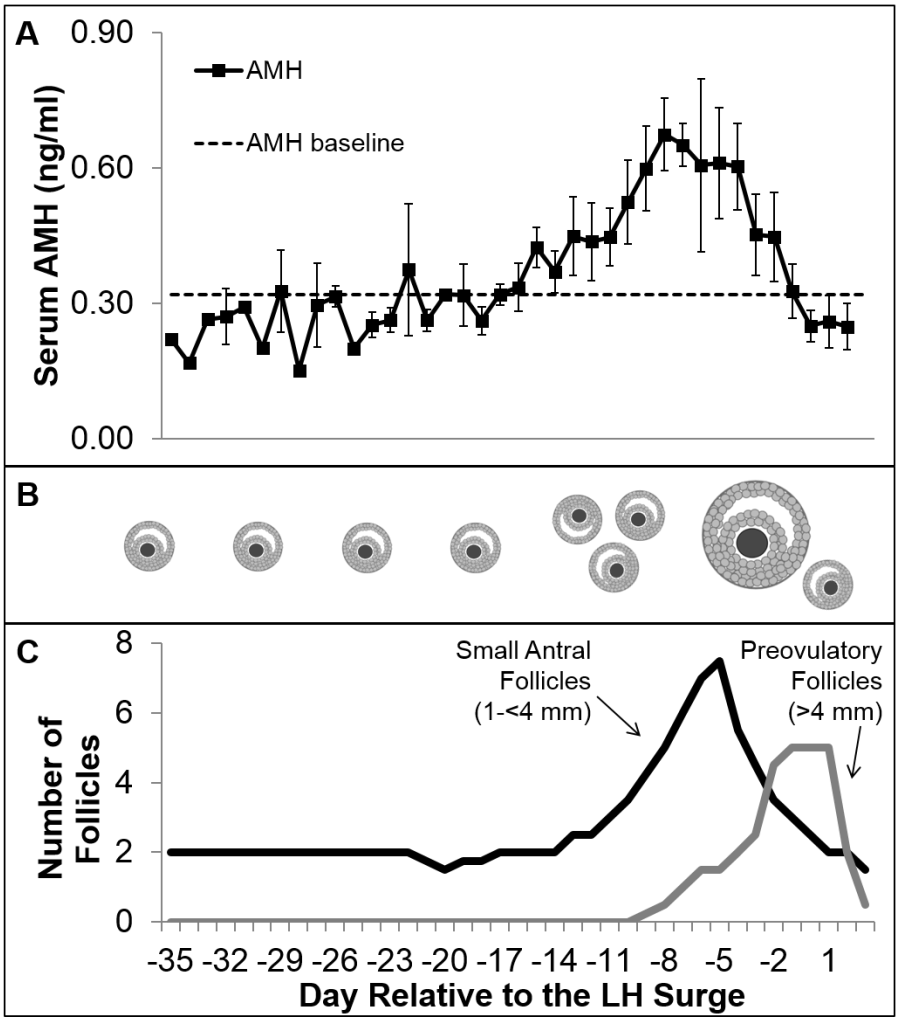

Figure 4.2: Comparison of mean serum AMH concentrations (± SEM) to large follicle population trends in the ovary during the transition from anestrus through estrus. A) Mean serum AMH concentration (N=5) from days -35 to 3. The dashed line indicates the mean baseline AMH concentration. B and C) Schematics of early antral (1 to <4 mm) and pre-ovulatory (>4 mm) follicles observed via ultrasound, adapted from England et al., 2009

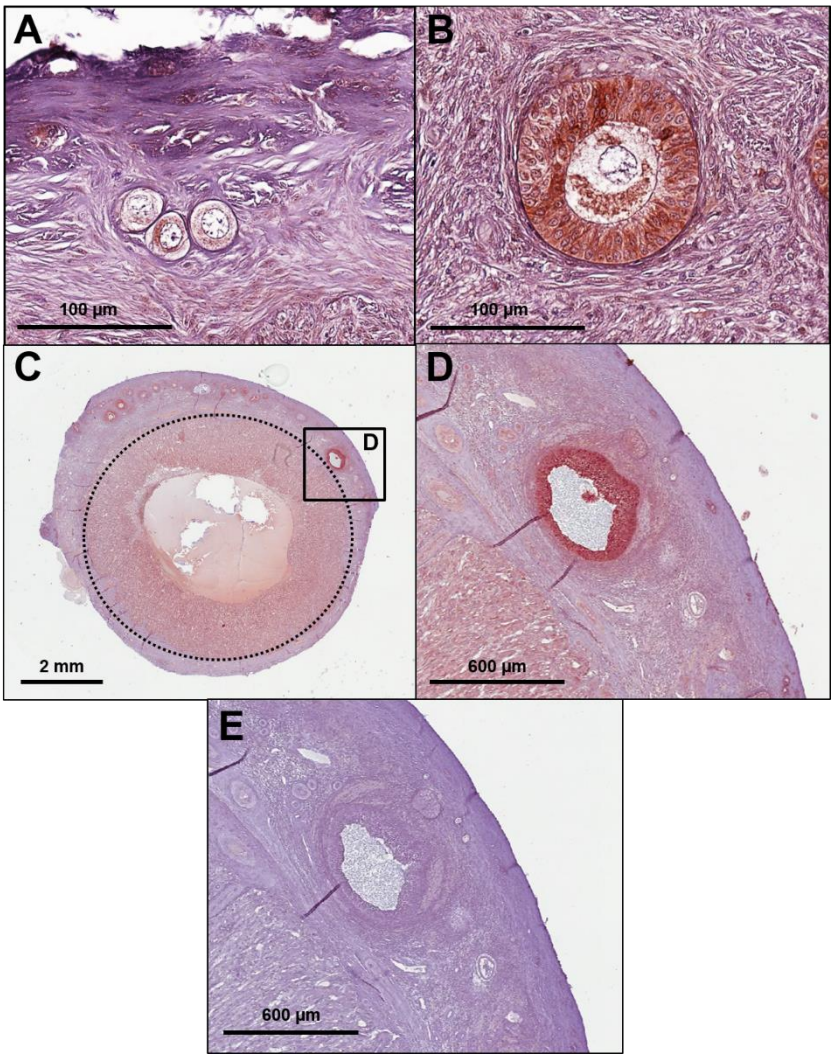

Figure 4.3: Representative photomicrographs of AMH immunohistochemistry. A) primordial follicles from cortical tissue, B) preantral follicle, C) large pre-ovulatory follicle (dashed black circle), D) antral follicle from box in (C), and E) Negative IHC control (no primary antibody).

Although considerations must be taken in sample collection timing, AMH could prove useful in the development of assisted reproductive technologies in canids. In the domestic dog, oocytes are ovulated at the germinal vesicle stage and require an additional 48-72 hours in the oviduct to reach metaphase II (MII) and become fertilizable [5]. Attempts to recapitulate this maturation process *in vitro* have generally yielded low rates of MII development and subsequent

in vitro fertilization embryo production [43-47]. No live births in dogs have resulted from IVM/IVF efforts to date. The ability to predict the date of ovulation 10 days in advance by monitoring AMH concentrations could facilitate improved collection of preovulatory oocytes to probe for mechanisms of meiosis resumption that are potentially unique to canids. Anti-Müllerian hormone could also provide more advanced notice than LH and progesterone monitoring for scheduling breeding opportunities and optimal artificial inseminations as part of canid conservation efforts. However, AMH testing has limitations and further work needs to be done to optimize blood sample collection frequency to establish the anestrous baseline and the most predictive rise. Additionally, it remains to be seen if the present study's results are applicable to other canid species, especially those for which more advanced assisted reproductive technologies might be used as part of conservation efforts. For example, frequent blood sampling is not practical for wild species, therefore, less-invasive methods using fecal or urine samples will likely need to be developed. Inhibin, another member of the TGFβ superfamily, has previously been assessed as a diagnostic test in the urine of women [48], which encourages the possibility that AMH may be non-invasively measured in the future.

Moving forward, if AMH is found to correlate with the number of ovarian follicles in the dog, as it has in other species, insight into the size of the ovarian reserve would greatly aid *ex situ* endangered canid conservation efforts. This information could help to inform decisions about best breeding pair and population planning with aging individuals. Additionally, domestic dogs have historically responded with high variability to estrus induction protocols [49] and improved rates of response have been noted in late anestrus dogs compared to early and diestrus animals [50, 51]. If the correlation between AMH values and antral follicle counts is confirmed in dogs, as the results of this study suggest, it is possible that AMH might prove to be a useful marker that predicts the response to estrus induction treatments, which would be similar to its utility in women [11]. Monitoring AMH concentrations in canids might allow investigators to better determine which animals will best respond to estrus induction protocols.

In summary, the present study resulted in three major findings. 1.) An elevation in serum AMH concentration two-fold above baseline appears to predictably predate the LH surge by 8-9 days. 2.) AMH values are variable among similarly aged dogs, all of which were sampled during their peak reproductive years (2.5-4.0 y). 3.) The most likely source of AMH in domestic dog circulation is the granulosa cells of preantral and antral stage ovarian follicles. Future work should determine if serum or urinary AMH concentrations correlate with the size of the ovarian reserve in dogs and if an individual dog's AMH concentration during proestrus is predictive of her response to ovarian stimulation.

Acknowledgements

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CHAPTER FIVE

In Vitro Fertilization of *In Vivo*-Matured Domestic Dog (*Canis familiaris*) Oocytes: Effects of Collection Day, Media Composition, Progesterone Supplementation and Male Donor*

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* Nagashima, J.B., Sylvester, S.R., Nelson-Harrington, J.L., Cheong, S.H, Songsasen, N., Travis, A.J. *In Vitro* Fertilization of *In Vivo*-Matured Domestic Dog (*Canis familiaris*) Oocytes: Effects of Collection Day, Media Composition, Progesterone Supplementation and Male Donor. *Manuscript in preparation*

Abstract

Development of assisted reproductive technologies in the dog has lagged behind other species, despite their potential importance for both conservation of endangered canid species and advancement of the domestic dog as a biomedical model. Here we describe the first live births from *in vitro* fertilized embryos in the domestic dog. Utilizing improved sperm capacitation protocols and *in vivo* matured oocytes, we evaluated the effects of collecting oocytes on various days post luteinizing hormone (LH) surge, culture media composition, progesterone supplementation, and male donor, on *in vitro* embryo development. Oocytes collected six days after the LH surge, fertilized and cultured in an NCSU-based medium with essential and nonessential amino acids, resulted in high rates of embryo development (78.5%, n = 144). Embryos cultured to the 96 hr mark (Day 10 post-LH surge) readily developed to the 8-cell stage (71.4% of 21 embryos). Out of 34 oocytes cultured past 96 hrs, we did not observe development beyond the 16-cell stage, suggesting that an *in vitro* developmental block exists at a later point than the time of embryonic genome activation in the dog. There was no effect of progesterone supplementation on embryo development, whereas a significant donor male effect was observed. A total of 65 four- to eight- cell embryos were transferred fresh or after vitrification/thawing to five naturally cycling recipient bitches. In the first 4 females, we transferred into the proximal uterine horn. Fetal sac development was visible in 3 recipients via ultrasound, but no live births resulted. In the fifth female, we performed embryo transfer into the oviduct. Out of 19 embryos transferred, 7 live pups were born, representing the first pups produced by IVF in the dog.

Introduction

Despite the rapid advancement of assisted reproductive technologies (ART) in human medicine over the past several decades, development of similar techniques for the domestic dog has remained stagnant. For example, *in vitro* fertilization (IVF) first resulted in a human birth in 1978 [1], but no live pups have ever been produced by IVF in the dog. This surprising lack of progress now represents a critically important stumbling block on two main fronts. First, given the continuing decline of populations of multiple canid species worldwide [2], there is an urgent need to use ART to maintain genetic diversity in captive populations and establish insurance populations as a hedge against extinction. Second, there is growing interest in utilizing the domestic dog as a biomedical research model, particularly as it shares many genetic diseases and exposure to the same environmental factors common to humans [3]. To realize this potential, we must be able to precisely control canine reproduction for colony management, as well as for the development of specific transgenic models through use of ART. Although several studies have demonstrated differentiation of putative dog embryonic stem cells into all three germ layers [4, 5], none have reported successful germline transmission. Therefore this lack of true embryonic stem cells in this species focuses our attention on technologies such as CRISPR [6], which would be greatly facilitated by IVF.

There are several aspects of the dog's reproductive biology that have contributed to the slow progress of ART for this species. Dogs experience a prolonged, variable period of ovarian inactivity or anestrus [7], and as result ovulation only occurs once or twice annually. Canid oocytes are ovulated at an immature stage compared with other mammalian species (primary oocyte, pre-germinal vesicle breakdown), and require 48 – 72 hrs in the oviduct post-ovulation to complete nuclear maturation [8]. There is little understanding regarding the mechanisms of dog oocyte maturation *in vivo*, and the combination of these factors means there is a paucity of mature oocytes available for development of technologies such as IVF.

Due to these constraints, previous attempts at canine IVF have used *in vitro* matured oocytes collected at various stages of the reproductive cycles, and often from ovarian follicles in various stages of development. *In vitro* maturation, or IVM, generally has resulted in low rates of successful resumption of meiosis, with the exception being one study that found that use of oocytes from follicles larger than 2 mm yielded significantly higher rates of metaphase II development *in vitro* [9]. When paired with IVF, these previous attempts report low rates of embryo production (2.2%-33.6%), and have only produced 1 blastocyst [10] and 3 morulae [11-13] out of hundreds of oocytes. As understanding of oocyte maturation is lacking in the dog, we thought it likely that these IVF attempts failed due to poor quality or developmental capacity of oocytes that have been matured *in vitro*. To eliminate the variability of *in vitro* matured oocytes in fertilization studies, *in vivo* matured oocytes could be utilized. *In vivo* studies evaluating maturation status of post-ovulatory oocytes suggests that the optimal time to collect mature dog ova is at least 3 days after ovulation, or Day 5 post-LH surge [8]. However, the fertilization window for domestic dogs has been demonstrated to extend beyond this time point, as viable pregnancies via intrauterine AI have been produced from oocytes up to 10 days after the LH surge [14]. Therefore, optimal time for mature, fertilizable oocyte collection in the domestic dog is not yet known.

It is also possible that IVF in the dog has not been successful due to either a sperm or embryo culture condition defect. We therefore re-assessed protocols for canine sperm capacitation and discovered that magnesium helps promote calcium flux and downstream functional endpoints such as hyperactivation of motility and ability to undergo induced acrosome exocytosis (Sylvester et al., manuscript in preparation). In the current study, we also assessed increasingly complex culture media. This was done to rule out a potential component in the medium blocking embryo development, as in the hamster [15], or a necessary component for development beyond embryonic genome activation block experienced by most species *in vitro* (e. g., bovine [16], ameliorated with amino acids [17]). Two base media were evaluated in our initial pilot studies:

synthetic oviductal fluid (SOF) and North Carolina State University medium (NCSU), the latter with varying complexity via supplementation of amino acids.

We also assessed the impact of donor male and progesterone supplementation on fertilization and embryo culture. In human IVF, there have been conflicting reports regarding the effect of donor male on embryo outcome, particularly with regard to aging males [18-20]. We were interested to know if there was an effect of donor on embryo production rates when using proven breeder males. Regarding progesterone, *in vivo,* circulating and local progesterone levels begin to rise prior to the LH surge and subsequent ovulation (preovulatory luteinization) [21]. Therefore, the hormonal environment the fertilizing oocyte and early embryo are exposed to is high in progesterone.

We hypothesized that *in vivo* matured oocytes collected Day 5 post-LH surge would be fertilizable in minimally-supplemented medium, that progesterone addition would improve rates of embryo development, and minimal influence of donor male would be observable. In the current study we determined that: 1) Day 6 post-LH surge was optimal for obtaining developmentally competent oocytes, 2) a minimally supplemented medium supports the fertilization of *in vivo* matured oocytes, 3) more complex culture media was not required for embryos to develop past the stage of embryonic genome activation, 4) there was no significant effect of progesterone on embryo production, and 5) male donor significantly influences oocyte fertilization and embryo development in the dog.

Materials and Methods

Animals and estrus/ovulation monitoring

Forty-eight proven-breeder beagles (aged 1.5 – 5 years) from Marshall BioResources (North Rose, NY) were monitored from natural (unsynchronized) late anestrus through estrus. Animals were group housed on a 12 hr/12 hr light/dark cycle and fed once daily laboratory canine diet #5006 (LabDiet, St. Louis, MO) and provided water *ad libitum*. All experimental

protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University.

Blood was drawn alternately from right and left cephalic and saphenous veins 3 – 7 days a week, with increased frequency to daily sampling once proestrus was suspected via either presence of serosanguinous discharge from the vulva and/or serum progesterone values higher than 0.4 µg/ml. Collected blood was allowed to clot, then centrifuged at 700g for 10 min to separate serum which was evaluated via chemiluminescent immunoassay (Immulite, Diagnostic Product Corporation, Los Angeles, CA), previously validated for use in the domestic dog [22] at the Animal Health Diagnostic Center at Cornell University. The days of the LH surge (day 0) and ovulation (day 2) were identified based on progesterone values of 1.5 – 2.5 and 4.5 – 5.5 ng/ml, respectively [22, 23].

Gamete/embryo collection and handling

Canine capacitation media CCM (CCM), was modified from Mahi and Yanigamachi [24]. CCM was supplemented with Hepes (25mM) as a buffer and 2.1 mM magnesium chloride (Sylvester et al, manuscript in preparation).

Five proven-breeder male dogs (Labrador retriever, Labrador-beagle mix, beagle, beagle-cocker spaniel mix, and cocker spaniel), aged 2-10 years, were used as semen donors. The first and second sperm-rich fractions were collected by manual stimulation following a minimum abstinence period of 2 days. The semen was centrifuged at 100 g for 1 min to remove debris, then diluted in CCM to wash before a second centrifugation at 400g x 5 min to pellet the sperm. Sperm were re-suspended in modified CCM then incubated in PVA-coated tubes at a concentration of 7.5 x 10^6 sperm/ml at 38˚C with 5% CO_2 in air for capacitation. Sperm were capacitated 2.5 – 3.5 hrs and then assessed for motility and hyperactivated motility.

Spays were performed by the Cornell Center for Animal Resources and Education veterinarians. On day 4, 5 or 6 post-LH surge, dogs were spayed and entire tracts immediately transported to the laboratory in warmed PB1 [25, 26]. Oviducts were dissected out of the bursa

and flushed with PB1 using a 23 G winged infusion set (Teruma Medical Products, Elkton, MD) connected to a 20 ml Leur lock norm-ject syringe. Oocytes were washed and IVF and embryo culture was performed in one of four media: base NCSU [27] or SOF [28] + 0.33 mM sodium pyruvate + 100 U/ml penicillin + 50 µg/ml streptomycin, mNCSU (base supplemented with 1% Basal Medium Eagle (BME) Amino Acids (Sigma Aldrich, St. Louis, MO) and 0.05 mM cysteine), or cNCSU (base medium with 1% BME and 1% Minimum Essential Medium (MEM) Non-essential Amino Acids solution (Sigma). Only high quality oocytes with heterogeneously dark cytoplasm [9] and normal morphometry (spherical, intact basement membrane) were utilized for the study. In most cases, the high lipid content and dense layer of cumulus cells surrounding post-ovulatory dog oocytes [8] obscured our ability to observe an extruded polar body. Oocytes were washed in medium before transfer to fresh, pre-equilibrated 100 µl droplets of the various media overlayed with mineral oil for IVF.

In vitro capacitated sperm were then added to the oocytes in the 100 µl IVF droplet at a final concentration of 1 million sperm / ml. The gametes were co-incubated for 14 hrs at 38ºC and 5% CO_2, 5% O_2 and 90% N_2 in a humidified hypoxia chamber.

After 14 hrs, presumptive zygotes were washed by gentle pipetting in fresh medium to remove loosely attached sperm and/or cumulus cells, then transferred to individual, 50 µl droplets, pre-equilibrated in mineral oil. Images were taken with Spot Software 4.1 (Diagnostic Instruments, Sterling Heights, MI). Presumptive zygotes were returned to culture (medium dependent on study – see “Experiments” section) under the same temperature and gas conditions. Embryo cleavage was evaluated at 48 hrs post-IVF, then embryos were either cryopreserved or medium exchanged for further culture, re-evaluating cleavage stage every 24 hrs subsequent (72, 96 hrs).

Embryos were vitrified and thawed using commercially available Vit Kit Freeze and Thaw (Irvine Scientific, Santa Ana, CA) according to manufacturer instructions. Cryopreserved embryos were allowed to recover in Medium 199 supplemented with 20% fetal bovine serum (v/v) for 2-3 hrs prior to transfer into five recipient beagles experiencing natural estrous cycles on the

appropriately synchronized day (8 or 10 post LH surge). Embryos were transferred in groups based on IVF progesterone treatment group.

For Transfers #1-3, embryos were frozen between 48-55 hrs post IVF (or Day 8 post LH surge) and transferred into the uterine horn. Embryos frozen between 94-98 hrs post IVF (or Day 10) were also transferred to the uterine horn for Transfer #4. In the 4th transfer, we hypothesized that there might be some undescribed maternal recognition of pregnancy. We therefore performed artificial insemination with 1 ml semen from a 3 yr old Labrador donor on day 3 prior to the transfer on day 10. For uterine horn transfer procedures, a stab incision was made into the cranial portion of the uterine horn, exposed via laparotomy, and embryos in minimal medium (Medium 199 + 20% FBS, 15-40µl) were gently pipetted into the lumen using a manually-truncated 200 µl pipette tip. In Transfer #5, Day 8 frozen-thawed embryos were transferred into the oviduct of the recipient. For oviductal transfer, both left and right ovarian bursas of a single recipient hound experiencing a natural estrous cycle were exteriorized via laparotomy. The suspensory ligament was manually dissected and a slit was made through the bursal window to better access the infundibulum. Embryos were drawn up into a 3.5 FR, 4.5" (11.4 cm) tom cat catheter (Argyle, Covidien, Mansfield, MA) with a 1 cc syringe in 20 µl medium, then the catheter was advanced approximately 2.5 cm into the oviduct via the infundibulum and the embryos were slowly dispensed. Pregnancy diagnosis and checks were performed via ultrasound weekly beginning Day 30 post LH-surge, and all births were via C-section on Day 65.

Experiments

Pilot study 1

In the first pilot study, oocytes were collected from bitches (N = 13 dogs) on either Day 4 or Day 5 post LH surge. The 14 hr IVF using sperm capacitated in the absence of magnesium took place in a droplet of Medium 199 (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA). Presumptive zygotes were transferred to embryo culture media as described above. For oocytes collected on Day 5 (N = 12 dogs, n = 76 oocytes),

four embryo culture media were evaluated: SOF (n = 13), NCSU (n = 15), mNCSU (n = 11), and cNCSU (n = 37).

Pilot study 2

Oocytes were collected from two bitches on day 6 post-LH surge, and media composition of IVF and embryo culture droplets were the same as in Pilot study 1. IVF was carried out in either SOF (n = 3), NCSU (n = 2) or cNCSU (n = 2).

Study A

Day 6 oocytes (n = 144) from 24 dogs were collected and IVF/embryo culture was carried out exclusively in cNCSU, based on results of the two pilot studies. Half the oocytes collected from each individual were also exposed to 6 µg/ml progesterone (in DMSO, Sigma) added to the cNCSU for both IVF and embryo culture. Embryos produced in this study were either vitrified for later embryo transfer into one of five recipient dogs, transferred "fresh" without freezing, or cultured until first appearance of degeneration.

Statistics

Pilot studies involving small numbers of animals and oocytes were used to make rough comparisons among media and day of collection. We have therefore excluded them from statistical analysis of the main study. Chi square / likelihood ratio test were used to determine differences between variables (P4 supplementation, male sperm donor) on embryo development rates using JMP 10.0 Software (SAS, Cary, NC)

Results

Pilot Study 1

No embryos were produced with Day 4 oocytes (N = 1, n = 6), although embryo development was observed in oocytes flushed Day 5 post LH surge (Table 5.1). Within these, we evaluated four increasingly complex media. No embryos were produced using these Day 5 oocytes in the two most basic media, SOF and NCSU, nor in mNCSU, although, in the latter

medium, two were observed to cleave after the expected 48 hr post-IVF mark (henceforth referred to as “delayed cleavage”). A total of 11 embryos were produced from 37 oocytes in cNCSU (29.7%), although rates of delayed cleavage were similarly high in this culture medium (Table 5.1).

Collection Day (post LH surge)	# Oocytes	Culture Medium	Normal Cleavage (%)	Delayed Cleavage (%)	Total Embryo Production (%)
4	5	mNCSU	0 (0)	0 (0)	0 (0)
5	5	SOF	0 (0)	0 (0)	0 (0)
5	5	NCSU	0 (0)	0 (0)	0 (0)
5	11	mNCSU	0 (0)	2 (18.2)	2 (18.2)
5	37	cNCSU	5 (13.5)	6 (16.2)	11 (29.7)

Table 5.1: Summary of Pilot Study 1 results with oocytes collected Day 4 or 5 post-LH surge and cultured in various media. “Delayed cleavage” refers to embryo development that occurred more than 48 hrs past IVF, or Day 7 post-LH surge.

Pilot Study 2

This pilot study exclusively utilized oocytes flushed 6 days post LH surge in three media. In NCSU (N = 1), 2/2 oocytes cleaved, resulting in one 8-cell, one 4-cell embryo. In SOF (N = 1), 2/3 oocytes became 8-cell embryos, and in cNCSU (N = 1), both oocytes developed into 8-cell embryos. Based on these data and cleavage results in Pilot Study 1, cNCSU was chosen as the primary culture medium.

Study A

Oocytes collected from bitches on day 6 of their cycle that were fertilized and cultured in cNCSU experienced very high rates of embryo development, with a total of 113 embryos produced from 144 high quality oocytes collected (78.5%). A small percentage of the total had delayed embryo development (n = 8 embryos, or 5.6% of the total).

No significant effect ($P > 0.05$) of progesterone supplementation on embryo cleavage was observed. Of the 42 oocytes cultured in the absence of P4, 32 successfully fertilized and

developed (76.2%), whereas 81 of 102 (79.4%) did the same in the presence of P4 (Fig. 5.1a). Few oocytes cleaved after the 48 hr mark in either treatment, and the impact of presence/absence of progesterone was not statistically significant (Fig. 5.1b). Only a portion of normally-cleaving embryos (N = 6, n = 21) were cultured to the 96 hr mark, or Day 10. However, rates of 8-cell embryo development were high (71.4% of normally cleaving embryos, or 44.1% of total oocytes cultured for 96 hrs). Images of an individual oocyte through collection, IVF, and 96 hr culture are shown in Fig. 5.2a-d. In one female, embryos (n = 5) did not cleave until the 72 hr mark. These were also cultured to the 96 hr time point, however they did not advance beyond the 2- and 4-cell stages.

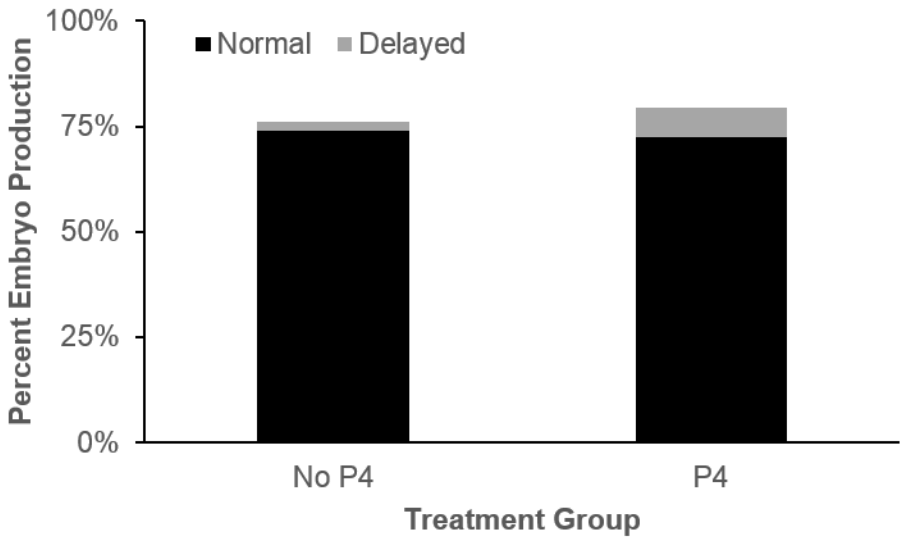

Figure 5.1: Proportion of oocytes collected on Day 6 developing into embryos in un-supplemented versus progesterone-supplemented cNCSU, with comparisons of oocytes cleaving normally (by 48 hrs of culture) in black, and experiencing delayed cleavage (after 48 hrs) in grey.

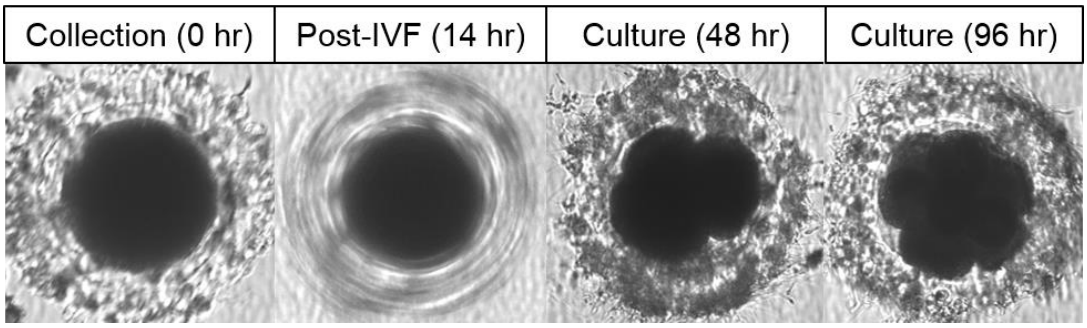

Figure 5.2: Images of an individual oocyte at collection though fertilization and 96 hr culture developing into an 8-cell embryo. A focused image of the presumptive zygote immediately following the IVF period (14 hr mark) was not possible due to motility of attached sperm.

A male effect was noted for one male regarding total fertilization success and another male for delayed-cleaving embryos (Fig. 5.3 – data exclude trials done with multiple sperm donors). Regarding overall cleavage rates, a sperm donor effect was noted for a 10 year old Labrador retriever who was retired from sperm collection over the course of this study. While there were no visible abnormalities or obvious motility / capacitation differences in his sperm, only 9 / 20 oocytes fertilized with his sperm cleaved (45%), a significant ($P < 0.01$) contrast to results from his offspring, a two year old Labrador-beagle mix that produced 7/7 (100%) embryos. Exclusion of oocytes incubated with this 10 year-old Labrador increased rates of embryo development (total of 21/25 or 84% for no P4, and 71/80 or 88.8% for P4 treated), but made no difference statistically between treatment groups for normally cleaving embryos. However, significantly more ($P = 0.0466$) oocytes experienced delayed cleavage in the presence of progesterone in the dataset if calculated excluding this male, likely because the decrease in sample size emphasized the higher rates of delayed cleavage in oocytes fertilized by sperm from a six year old cocker spaniel (Fig. 5.3), who was responsible for the second male effect noted.

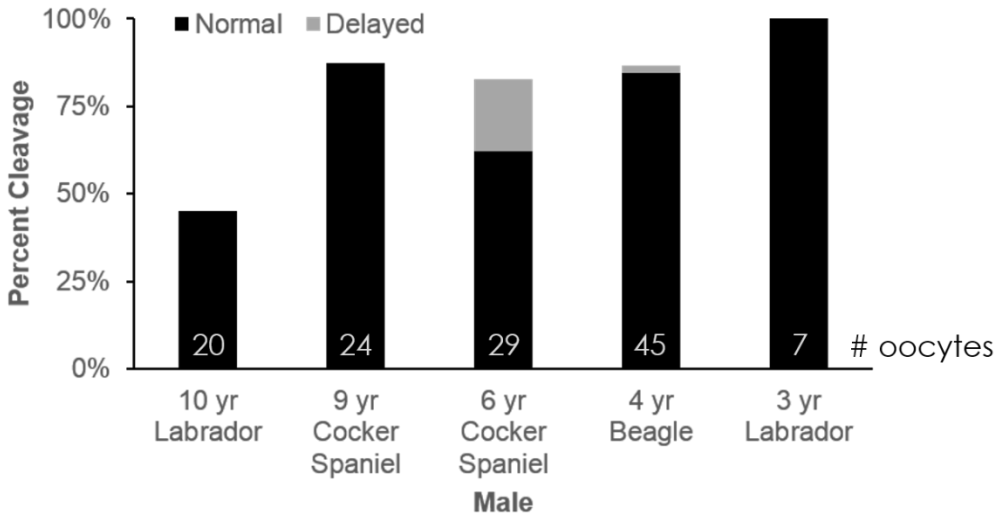

Figure 5.3: Effect of male sperm donor on embryo cleavage rates displaying proportions of oocytes undergoing normal cleavage (black bar, $P < 0.05$), and those which cleaved beyond the 48 hr mark (grey bar).

Attempting to generate offspring, we performed transfer with both fresh and frozen embryos into naturally synchronized females are described in Table 5.2. IVF-derived embryos were initially transferred into the uterine horn of recipients (Transfers #1-4). Although implantation and fetal sacs were observed, no live pups were born. In Transfer #5, embryos were put into the lumen of the oviduct via cannulation of the oviduct via the infundibulum. A total of seven gestational sacs were visible via ultrasound Day 29 (Fig 5.4a) and 41 (Fig 5.4b) post-LH surge, resulting in seven live offspring (3 females, 4 males from two separate sperm donors) being born by planned C-section (Fig 5.4c).

Transfer #	# Embryos Transferred	Embryo Stage	Status	Transfer Location	Results
1	4	2-4 cell	Fresh	Uterine Horn	Gestational sac, no visible heartbeat
2	10	4-8 cell	Frozen-thawed	Uterine Horn	Gestational sacs, possible heartbeat
3	23	4-8 cell	Frozen-thawed	Uterine Horn	No evidence of implantation
4	9	≥ 8 cell	3 frozen-thawed, 6 fresh	Uterine horn of AI'd recipient	Only AI-derived offspring
5	19	4 cell	Frozen-thawed	Oviduct	7 offspring born

Table 5.2: Embryo status, transfer location and result of IVF-derived embryo transfers

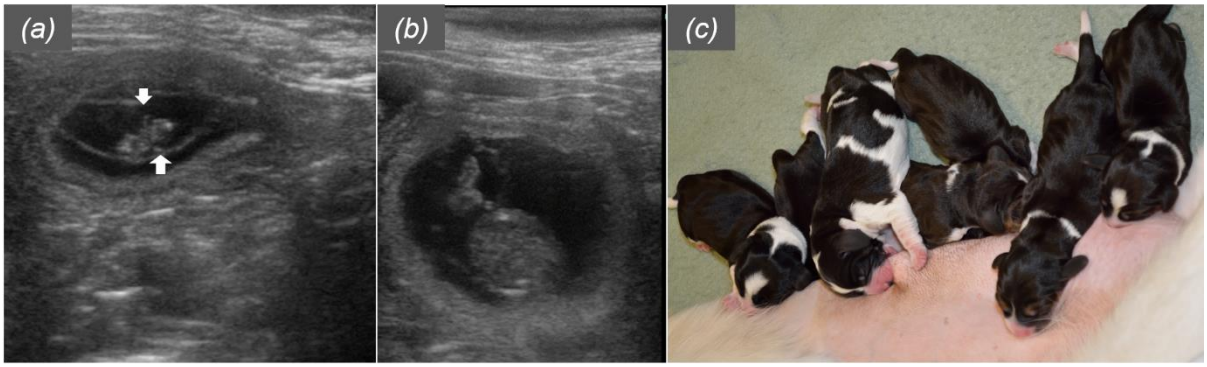

Figure 5.4: Ultrasound images, Transfer #5. *(a)* a normally-developing embryo imaged Day 29 post-LH surge, with white arrows indicating limb buds, *(b)* a gestational sac on Day 41, and *(c)* 7 healthy puppies born by planned C-section.

Discussion

Attempts at IVF in the dog have previously resulted in very low rates of embryo development and no live births. We initially hypothesized that this lack of success was due to prior use of *in vitro* matured oocytes of unknown developmental competence. This guided our experimental design to determine what day post-ovulation was optimal for obtaining

developmentally competent oocytes, and then determine what media and conditions were optimal for fertilization and early embryo development in the dog.

Utilization of Day 6 *in vivo* matured oocytes and an improved sperm capacitation protocol (Sylvester et al, manuscript in preparation) enabled us to consistently produce embryos at rates/proportions comparable to that of the laboratory mouse. Results of our pilot studies indicated that day of oocyte collection was critical to these success rates under the conditions we employed. Although studies of nuclear status of *in vivo* matured oocytes have determined canine oocytes resume meiosis and reach metaphase II by 72 hrs post ovulation (Day 5) [8], oocytes collected at this stage performed relatively poorly in our IVF system. However, dogs have been shown to experience a relatively long window of fertilization, wherein oocytes up to 10 days after the LH surge have produced viable pregnancies via intrauterine AI, with high rates of pregnancy success with AI performed at days 7 and 8 [14]. Therefore, although nuclear maturation might be completed by Day 5 *in vivo*, this does not necessarily correspond to the optimal time for fertilization. Our data suggest additional maturation steps are needed in dog ova for peak fertilization rates. Some aspects of cytoplasmic maturation are tightly coupled with nuclear maturation, as the former is required for the extrusion of the polar body [29]. For example, the microfilament actin, as well as actin nucleators actin-related protein 2/3 complex [30] and formin-2 [31], have been shown to be critical to the migration of chromosomes toward the cortex prior to polar body extrusion during meiotic maturation in the mouse. Based on improved understanding of cytoplasmic maturation in other species [32], it could be informative to determine whether aspects of cytoplasmic maturation might not be supported by current *in vitro* dog oocyte maturation protocols.

Our data demonstrated that basic media (minimally supplemented NCSU, SOF) were sufficient for fertilization (Pilot Study 2), provided the oocytes were collected at Day 6. Synthetic oviductal fluid (SOF) is currently used in many human and bovine studies but originally was developed to mimic the oviductal fluid composition of the sheep [28]. North Carolina State

University medium (NCSU) designed for porcine oocytes [27], was also evaluated because the lipid content of dog oocytes is similar to those of the pig, particularly with regard to phospholipid content [33, 34]. Our modified cNCSU provided more flexibility in oocyte collection times, evidenced by its higher rates of embryo development even in oocytes collected on Day 5 post-LH surge. This flexibility was important because our hormone monitoring protocol allowed variances of up to 12 hr in the timing of an individual bitch's LH surge.

Surprisingly, P4 supplementation had no beneficial effect on embryo cleavage. Both the ovarian follicle [35] and the oviduct [36, 37], display increasing levels of P4 receptor during the periovulatory period in the dog. Recent evaluations of the composition of canine oviductal fluid demonstrated high levels of P4 [38]. This led us to hypothesize that progesterone has a role in oocyte maturation through early embryo development. Our data do not rule out whether this hormone plays one or more functions in the periovulatory canine oviduct, on either the immature oocyte and/or the oviductal epithelium, and/or the sperm. For example, exogenous P4 can be affects Ca^{2+} influx, capacitation and acrosome exocytosis in murine sperm [39], and we used it to induce Ca2+ transients facilitating acrosome exocytosis in dog sperm (Sylvester et al, manuscript in preparation). That being said, we saw no evidence for an effect of P4 to promote fertilization of day 6 oocytes.

Conversely, there was anecdotal evidence that more oocytes experienced delayed cleavage in the presence of P4. It is uncertain if this delay was beneficial (rescue effect) or detrimental (indicative of poor developmental competence). Knowledge of reproduction in canids is still remarkably behind that of other mammalian species, so more research is needed before speculating whether P4 has effects on the oocytes, sperm, or embryos prior to cleavage. Indeed, several studies in the domestic dog have noted a relatively wide distribution of early embryonic stages in the oviduct post-fertilization [8, 40, 41], suggesting this variation is not necessarily a product of the *in vitro* culture conditions. Consistent with this, we observed that in

some cases the late-cleaving oocytes ‘caught-up’ developmentally with the other embryos in their cohort.

The 8-cell stage is the stage of embryonic genome activation in the dog [42]. In our system, embryos cultured beyond the 4-cell stage reached the 8-cell stage readily. The few that we cultured past the 96 hr mark did not develop beyond the 16-cell stage; this has been reported both in other studies of canine IVF [43], and even when culturing *in vivo*-derived dog embryos [44]. Together, the data suggest an *in vitro* developmental block at a later time point than embryonic genome activation. To circumvent this, we transferred embryos produced to recipient uteri at the 4-cell stage. Success in terms of both implantation [45] and live birth [46] has been shown in the transfer of *in vivo*-derived early stage embryos into this portion of the reproductive tract, although embryos do not reach the uterine horn until Day 11 post LH surge (~16-cell stage) *in vivo* [41]. As live fetuses with normal morphology and heart rate were observed via ultrasound up to Day 51 post-LH surge, this represents the most advanced stage of IVF-derived embryo gestational development to date.

Using fresh *in vivo*-derived embryos, Tsutsui et al reported 51.9% success rates in 8-cell to blastocyst stage embryo transfer [47]. In this laboratory, our work transferring vitrified 8- and 16-cell *in vivo*-derived embryos resulted in a 16% live birth rate using a closed vitrification system (Vit Kit, Irvine Scientific, Irvine, CA), and Abe et al reported 9.1% using the Cryotop method [26]. Transfer success rates for IVF-derived, vitrified embryos are likely to be low with currently available vitrification methods. The high lipid content in dog embryos is likely detrimental to the vitrification process [48], as was the case in the pig [49]. Development of delipidation protocols prior to vitrification may improve cryosuccess in the dog and, by extension, likelihood of successful frozen-thawed embryo transfer. However, the current study resulted in significant implantation success using VitKit in 4-cell stage embryos in conjunction with oviductal transfer (seven live births from total of 19 embryos transferred, or 36.8%),

suggesting that success rates may also be improved by more stage-appropriate transfer locations for early stage embryos.

Collection of specifically-timed, *in vivo* matured oocytes, and manipulation of culture media conditions, combined to yield not only very high rates of embryo development, but the first successful live births from IVF in the domestic dog. This study has identified a consistent, reproducible protocol for IVF—an urgently needed step in the development of additional ART for dogs. This advancement not only provides a reliable standard of comparison to evaluate the efficacy of *in vitro* maturation protocols, but also represents a significant step forward in our ability to utilize the domestic dog as a model in endangered canid reproduction and biomedical research.

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CHAPTER SIX

Final Discussions and Future Directions

Overview

Studies included in this dissertation describe advances made in the understanding of anestrus termination / estrus resumption, folliculogenesis, and fertilization in the domestic dog. This section provides a summary of these advances, as well as possibilities for future areas of investigation in the field.

<u>Ovarian Follicle Culture</u>: 1) Follicular Size/Stage and Gonadotropin Concentration Influence Alginate-Encapsulated *In vitro* Growth and 2) Survival of Pre- and Early-Antral Dog Follicles and Activin Promotes Growth and Antral Cavity Expansion in Domestic Dog Early Antral and Antral-Stage Ovarian Follicles

These studies have resulted in several key contributions to the field of domestic dog ovarian follicle culture. Pre- and early antral stage follicles encapsulated in a three-dimensional alginate hydrogel grew and produced steroids in the presence of FSH, although addition of LH did not improve these metrics except in the absence of FSH. While LH has been shown to be important in antral cavity development in other species, it did not appear to have a beneficial effect for the domestic dog. Supplementation of activin to the culture medium promoted the maintenance of trans-zonal projections, oocyte health (in terms of intact chromatin status), and antral cavity expansion. Culture of these follicles in a more dynamic, fibrin-alginate interpenetrating network (FA-IPN) allowed the maintenance of these large cavities. However, though antrum expanded under these conditions, they didn't develop from preantral stage follicles, suggesting the first area for future consideration.

The mechanisms of antral cavity development are not well understood in any species. Hypotheses involving aquaporins actively transporting water into the center of the follicle [1], and versicans and other large molecules being secreted by granulosa cells to produce an osmolarity gradient for water movement [2] have been proposed. Once initiated, it appears that maintenance and expansion of the antral cavity is supported by the FA-IPN culture system.

However, to reach the goal of producing viable oocytes from primordial stage dog follicles, this initiation of the antral cavity must be better understood. Additional antrum-promoting factors, such as vascular endothelial growth factor [3], may work in concert with factors like activin to initiate antrum formation. Further, for continued growth beyond evaluations made in these studies, these expanded antral stage follicles will likely require a great deal more nutrients and energy substrates than their smaller counterparts. This is supported by *in vivo* conditions, wherein vascularization has been shown to develop progressively over follicle growth/development, particularly at the antral stage when dominance follicle status is determined [4, 5]. Therefore, we propose in the future that a more dynamic, microfluidic culture system might be utilized to improve transport of nutrients to these cultured follicles.

The earliest stages of folliculogenesis (primordial follicle activation, primary follicle development) were not addressed by this study. Work is being done to understand the species-specific regulators of primordial follicle activation using ovarian cortical tissue culture [6]. This is particularly critical as the ovarian cortex of the dog is notably more rigid than that of the mouse. It has been proposed that the interplay between the physical microenvironment provided by the cortical tissue and the early follicle regulate activation. There is one school of thought that one of the early stages of activation is the initiation of secretion of factors such as matrix metalloproteinases to ‘soften’ the cortical tissue to allow follicular growth [7]. Conversely, a permissible microenvironment may select which follicles are able to activate [8]. We identify this as a third area of focus for future studies regarding the improved understanding of domestic dog ovarian folliculogenesis.

<u>Anestrus Hormone Monitoring</u>: **Anti-Müllerian Hormone in the Domestic Dog during the Anestrus to Estrus Transition**

The temporal rise of circulating AMH during canine proestrus is a compelling topic for future studies. If this pattern holds true for a larger population, and, specifically, in a wider age

range of individuals, it has the potential to be a useful tool for the more accurate prediction of the onset of estrus or LH surge in our laboratory dogs. More specific and advanced notice of the LH surge would also be incredibly valuable for endangered canid breeding efforts; however, a critical next step to achieve this is to determine if AMH can be assessed non-invasively in these species (i.e. via urine or feces). AMH has been detected in neonatal human male urine [9], but at much lower levels than it should have been compared to serum in the same study, and another study evaluating serum values utilizing a commercially available kit [10]. However, another TGFβ superfamily member, inhibin has been identified in urine [11] suggesting that it may be possible to detect AMH noninvasively as well, particularly as tests become more sensitive.

Although not explored directly in this study, there is a known correlation between follicular reserve and serum AMH concentrations in other species, such as the mouse [12]. Future studies should evaluate if this relationship exists in canids as well, as it would be an important tool informing breeding pair decisions in endangered canids. For example, in the event the taxon advisory group is presented with an aging but genetically valuable female, it would be useful to know if it is worth the resource expenditure and stress on the animals to set her up as part of a new breeding pair for a natural mating, or if she is still a candidate for any ART for maternal genome rescue. Assessment of potential correlation between ovarian reserve and AMH in the domestic dog could be completed via opportunistic collection of ovaries from routine ovariohysterectomies, as a starting point. Ovaries from older animals might be obtained via retired breeders from various clubs/associations. If unknown, reproductive stage can be estimated by physical evaluation and assessment of structures on the ovary (i.e. presence/absence of corpora lutea, large antral follicles [13]), thus providing the ability to factor in high AMH values as result of proestrus. Serum from dogs in diestrus or anestrus, taken at time of spay, could be evaluated in parallel with discarded ovaries. Further, if ages are known for individuals, evaluation of the age-related changes in AMH could begin to be evaluated

(again, cautiously, as results of our study indicated variation in individual baseline). This analysis may be more difficult to assess in wild canids compared with domestic dogs, due to the seasonal reproduction of many canid species. In seasonally-breeding Siberian hamsters, AMH does not correlate with ovarian follicle reserve, as short-day, breeding females have more primordial follicles but significantly lower serum AMH compared to age-matched females in long-day [14]. This was suggested to be due to a difference in vascularization between the ovaries of hamsters in long day versus short day, but indicates a need to assess serum AMH concentrations and ovarian follicle populations in both breeding and non-breeding seasons for canids, to get an accurate evaluation of the use of AMH as a marker in seasonal canids.

Fertilization: *In vitro* Fertilization of *In vivo*-Matured Domestic Dog (*Canis familiaris*) Oocytes

Also critical to the advancement of the understanding of folliculogenesis in the domestic dog is *in vitro* fertilization. In the absence of a reliable protocol for IVF, there was previously no way to accurately test the viability of oocytes produced by *in vitro* folliculogenesis or oocyte maturation. Our IVF study yielded very high and reliable rates of embryo production, providing a consistent metric of comparison. Additionally, we determined that oocytes collected from bitches on Day 6 after the LH surge had the highest rates of embryo development, even in minimally-supplemented media. Dog oocytes resume meiosis and reach metaphase II between 48 and 72 hrs post-ovulation (Day 4-5 after the LH surge, but it is evident from our pilot studies that oocytes collected on these days do not have optimal fertility.

As dog ova are known to have extended windows of fertility (high pregnancy rates at Day 8 post LH surge via intrauterine AI [15]), it is likely that the timing of nuclear maturation does not necessarily correspond to time of peak fertilization ability. An important area of future study would be the evaluation of maturation, with particular emphasis on cytoplasmic maturation, in dog oocytes. Although cytoplasmic maturation is considered to be tightly linked

with nuclear maturation (e.g. cytoskeletal structures are responsible for the extrusion of the polar body), understanding of the cytoplasmic maturation requirements for fertilization and advanced embryo development are only recently being explored [16]. Considering how little is known about the mechanisms of meiosis resumption in dog oocytes *in vivo*, and how different the timing is compared to species like the mouse and human, it is probable that different requirements must be met for *in vitro* cytoplasmic maturation as well.

Our IVF study, as well as studies by Reynaud et al [17, 18], noted compact layers of cumulus cells associated with post-ovulatory oocytes, even to the 2-cell stage. In the mouse, the LH surge results in the disruption of cumulus cell connections with the oocyte, which result in a release from inhibition of meiosis [19]. While the LH surge has been shown to promote cumulus cell mucification in the dog, the inner 2-3 layers remain closely associated with the oocyte, and outer cumulus cell mucification is not associated with changes in chromatin configuration [17]. It has been suggested that this close association is related to the post-ovulatory delay in oocyte maturation in the dog [18], which is highly likely based on mechanisms described in other species, but still does not explain how maturation resumes in dog ova. Although these cells are closely associated, there is a possibility that the gap junctions between cumulus cells and the oocyte are disrupted, leading to meiosis resumption. To assess the functional relationship between oocytes and cumulus cells, researchers have previously used dye-transfer strategies, including the microinjection of Lucifer yellow into the oocyte and observance of transfer into cumulus cells [20], and FRAP experiments in which COCs loaded with calcein homodimer were laser-bleached to evaluate recovery of fluorescent signal [21]. This would be important to determine if the dense cumulus cell layer associated with post-ovulatory dog oocytes is directly communicating with the oocyte even after the LH surge. In turn, this would help us determine if the release from meiotic arrest is due to a factor in the oviductal fluid that is being transferred via the cumulus cells to the oocyte, or a factor acting in an LH-surge-like manner to disrupt COC communication after ovulation, or a different mechanism

entirely. Related to this, evaluations of the composition of dog oviductal fluid are currently underway [22, 23] and also represent a critical next step in determining the potentially unique mechanism of meiosis resumption in this species.

The evaluation of dog oviductal fluid potentially has an added benefit. Although development to the 8-cell stage (stage of embryonic genome activation [24]) was supported in our *in vitro* fertilization system, development beyond the 16-cell stage was not observed. Dog embryos remain in the oviduct until 11 days post LH surge [25], when they are at approximately the 16-cell stage. Insight into the hormonal and metabolic microenvironment the embryos are exposed to for this prolonged time would be important for the development of optimized culture media conditions.

The successful production of puppies via IVF described here also has huge potential for the development of specific biomedical models. A canine model expressing the human type 2 diabetes mellitus-related phosphoenolpyruvate carboxykinase (PEPCK) gene has been produced, which could be utilized to study the pathogenesis of this disease [26]. However, generation of models via somatic cell nuclear transfer is generally inefficient and, as result, prohibitively costly. More recently, the CRISPR/Cas9 system of transgenic animal development has found widespread use. Variations on the system continue to be developed, but in general a Cas9 protein and a ‘guide’ RNA is injected into a cell. The guide RNA has two functions. First, to recruit the Cas9 protein to the specific target in the genomic DNA. If successful binding occurs, Cas9 will make a double strand break in the DNA. Repair of this break either results in an insertion/deletion in the DNA, resulting in a disruption of the targeted gene, or, in the presence of a new template, will copy the new template sequence into the repair site. Use of this technology in an oocyte prior to fertilization or in a one-cell embryo allows for comparatively fast and inexpensive development of transgenic animal models, while maintaining much specificity and control of gene insertion/deletion/mutation. It has been utilized successfully in the mouse [27], pig [28], and monkey [29]. In the former, changes in multiple genes were completed in a

single step using several guide RNA sequences in concert, dramatically reducing the time and increasing the efficiency of transgenic model generation. Utilization of our highly efficient (>70% embryo production, 36.8% frozen-thawed, IVF-derived embryo transfer success) domestic dog IVF in combination with the CRISPR/Cas9 system would allow for more efficient development of transgenic animals for biomedical research.

Summary

The assessment and temporal rise in AMH during proestrus holds promise for future ART advancements in this species. Evaluation of the roles of FSH, LH, and activin on different stages of ovarian follicles contributes to our understanding of folliculogenesis, which, in turn, is necessary for the development of gamete rescue for endangered canid species. Finally, the first successful live births via IVF represent a major advancement of this ART in dogs, but also a critically needed tool for the evaluation of oocytes produced by other assisted reproductive technologies, such as *in vitro* follicle culture.

These discoveries described here represent significant advancements in the understanding of canid reproductive biology and hold potential for future application to the development/improvement of ART for endangered canid species.

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