THESIS

EVALUATION OF L-CARNITINE SUPPLEMENTATION DURING EQUINE OOCYTE *IN VITRO* MATURATION

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ABSTRACT

EVALUATION OF L-CARNITINE SUPPLEMENTATION DURING EQUINE OOCYTE *IN VITRO* MATURATION

Efficiency of *in vitro* embryo production in the horse remains limited, in part due to poor quality of *in vitro* matured equine oocytes. Equine *in vitro* maturation (IVM) is suboptimal, with only ~60% of immature oocytes maturing in culture, and those that mature often exhibit reduced quality compared with oocytes matured *in vivo*. A suboptimal culture environment likely contributes to these outcomes. Commonly used maturation media fail to recapitulate the *in vivo* maturation environment, lacking several metabolites present in follicular fluid. One such metabolite is L-carnitine, an essential cofactor for FAO. With emerging evidence that equine oocytes may rely on lipid metabolism for energy during maturation, L-carnitine therefore represents a potential strategy to improve IVM outcomes. The objective of this study was to determine whether L-carnitine supplementation during equine IVM enhances oocyte maturation, improves indicators of oocyte quality, and alters metabolic activity of the cumulus-oocyte complex (COC). Immature oocytes were recovered by transvaginal ultrasound-guided follicle aspiration and randomly assigned to standard maturation media, 2mM L-carnitine, and 4mM L-carnitine supplemented treatments. Following culture, oocytes were evaluated for nuclear maturation and cumulus expansion. Mitochondrial membrane potential, mitochondrial distribution, and reactive oxygen species (ROS) were also assessed using fluorescence microscopy. Alterations in metabolic activity were evaluated by GC-MS analysis of spent culture media. L-carnitine did not significantly alter maturation rates; however, cumulus expansion was

significantly enhanced in L-carnitine treated groups. ROS levels and mitochondrial distribution were not affected by treatment, while mitochondrial membrane potential was significantly reduced in the 4mM treatment as compared to standard maturation media. Analysis of spent media revealed increased lactate production in L-carnitine treated groups relative to fresh media, suggesting altered glycolytic activity during maturation. These findings represent the first evaluation of L-carnitine supplementation during equine IVM and suggest that L-carnitine may influence oocyte quality and COC metabolic activity during maturation, potentially improving *in vitro* embryo production outcomes.

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DEDICATION

To the horses in this study and to every oocyte we collected, mature or immature.

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CHAPTER 1: INTRODUCTION

Historically, the use of assisted reproductive technologies (ARTs) has been less successful in the horse as compared to other domestic species. The mare is seasonally polyestrous and typically ovulates a single follicle per cycle, limiting access to oocytes; this limitation is further compounded by a poor response to superovulation protocols [1]. Until recent advances, conventional *in vitro* fertilization has not been possible [2, 3]. As a result, *in vitro* embryo production has been made commercially viable in the mare by use of ovum pick-up (OPU) procedures, in which oocytes are collected from the live mare by transvaginal ultrasound-guided follicle aspiration (TVA), followed by fertilization via intracytoplasmic sperm injection (ICSI) [4]. Efficiency of OPU-ICSI programs rely on the retrieval of numerous immature oocytes, which must subsequently undergo *in vitro* maturation (IVM) [5]. IVM is the process by which an immature oocyte, arrested in meiosis, is cultured to achieve nuclear and cytoplasmic maturation allowing for fertilization. However, not all oocytes mature *in vitro*, with reported maturation rates of ~60% [6-9]. Suboptimal IVM results are likely attributable, at least in part, to incomplete understanding of the unique physiologic and metabolic requirements of the equine oocytes during maturation. Increasing evidence indicates that the equine oocyte exhibits unique metabolic characteristics compared with other species [10-11]. Reevaluating maturation conditions to better reflect the growing understanding of equine oocyte metabolism may enhance IVM and, in turn, expand clinical utility of ART in the mare.

Estrous Cycle of the Mare 1.1

The mare is a seasonally polyestrous, long-day breeder, with the physiologic breeding season extending from April through October in the Northern Hemisphere. During the breeding season, mares cycle repeatedly until pregnancy is established or the onset of fall transition. The estrous cycle averages 21-22 days in length and is comprised of two phases: estrus and diestrus [12].

Follicular development in the mare is driven by waves of follicle stimulating hormone (FSH) secretion from the anterior pituitary gland, which recruits cohorts of antral follicles initiating growth [13]. Although multiple follicles are stimulated each wave, the mare is monovular, with typically only one follicle selected for continued growth and ovulation [14]. As follicles grow, granulosa cells secrete increasing concentrations of estradiol-17β (E2) and inhibin, which act synergistically to suppress pituitary FSH secretion. [13]. The resultant decline in FSH is a key mechanism underlying follicular deviation and dominant follicle selection, as evidenced by the ability of exogenous FSH or anti-inhibin to induce development of multiple dominant follicles [1]. At the same time, rising E2 concentrations stimulate luteinizing hormone (LH) secretion, which supports continued growth of the dominant follicle and ultimately facilitates ovulation [12]. In addition, E2 induces characteristic changes within the reproductive tract, including increased uterine edema, and behavioral estrus [15]. Unlike other domestic species, the mare exhibits a prolonged rise in LH rather than a distinct surge, with ovulation occuring before maximal LH concentrations are reached. [16]. At ovulation, the dominant follicle ruptures, releasing the oocyte into the oviduct. This period, characterized by the dominant follicle and elevated E2, is the estrus phase (standing heat) of their cycle and typically lasts 5-7 days [12].

Following ovulation, granulosa and theca cells of the ruptured follicle undergo luteinization to form the corpus luteum (CL), a transient endocrine structure responsible for progesterone production during diestrus [12]. Progesterone exerts negative feedback on gonadotropin secretion, suppressing LH release and preventing development of another dominant follicle [17]. Despite reduced LH concentrations, FSH continues to be secreted in waves during diestrus, leading to recruitment of small antral follicles that undergo atresia in the absence of sufficient LH support [13]. In the absence of maternal recognition of pregnancy, the endometrium secretes prostaglandin F2α (PGF2α) between days 14 and 16 post-ovulation, inducing regression of the CL [18]. The resulting decline in progesterone removes negative feedback on LH secretion, permitting selection of a new dominant follicle. This process repeats until pregnancy is established or the mare transitions into seasonal anestrus [12-13]

Folliculogenesis 1.2

In mammalian species, oocyte meiosis begins during fetal or early postnatal life and is subsequently arrested until ovulation. By day 70-80 of gestation in the horse, oogonia have populated the fetal ovary, followed by significant degeneration of this population by days 100-120 [19]. Concurrently, primordial follicles form, characterized by a single layer of squamous granulosa cells surrounding the oocyte. The primordial follicles established in this time represent the ovarian reserve of oocytes that have initiated meiosis and subsequently arrested in prophase I [19]. Once an oocyte is recruited from the primordial follicle pool, it will progress through follicular development and ultimately be ovulated or undergo atresia. Recruited primordial follicles transition to primary follicles, characterized by granulosa cells becoming cuboidal and proliferative while the oocyte grows in size [20-21]. Concurrently, the oocyte becomes

surrounded by a thick glycoprotein layer known as the zona pellucida, which is critical for normal mammalian reproduction [22]. Together the oocyte and associated somatic cells produce signals that establish a highly regulated microenvironment necessary for proper follicular and oocyte maturation [23]. Communication between the oocyte and somatic cells occurs through both paracrine and direct cell-cell signaling via gap junctions. Two distinct granulosa cell populations exist: mural granulosa cells that line the follicular wall and cumulus cells, which directly surround the oocyte. Mural granulosa cells primarily respond to paracrine and endocrine signals within the follicular environment. On the other hand, cumulus cells have specialized gap junctions called transzonal projections that directly connect them to the oocyte, enabling efficient bidirectional signaling [24]. The bidirectional signaling between the oocyte and granulosa/cumulus cells is critical for coordinating oocyte growth and maturation, ultimately producing developmentally competent oocytes capable of fertilization and embryo development [25-26]. Follicles continue development from the primary stage with FSH support to produce secondary follicles characterized by stratified layers of granulosa cells. The follicle eventually forms a fluid-filled cavity called the antrum [27]. Antral follicles develop in distinct cohorts, or follicular waves, with one follicle becoming dominant over the others. Subordinate follicles undergo atresia, while the dominant (preovulatory) follicle continues growth and ultimately ovulates [13]. Final follicular development occurs concurrently with oocyte maturation, the process by which the oocyte completes meiosis I, acquires cytoplasmic maturity, and becomes competent for fertilization.

Oocyte Maturation 1.3

Nuclear Maturation 1.3.1

Oocyte maturation is most often characterized as completion of meiosis I and subsequent arrest in metaphase II, observed by extrusion of the first polar body. This process represents nuclear maturation and does not encompass the broad changes to the cytoplasm and organelles of the oocyte during maturation [28]. All oocytes begin this process arrested in the dictyate stage of prophase characterized by the presence of a large spherical nucleus referred to as the germinal vesicle [29]. Meiotic arrest of the oocyte is enforced by high concentrations of cyclic adenosine monophosphate (cAMP) within the oocyte produced by adenylyl cyclase 3. The oocyte is also able to break down cAMP by action of an oocyte specific phosphodiesterase; however, this phosphodiesterase is inhibited by the presence of cGMP produced by cumulus cells, which allows for continued meiotic arrest. Meiotic resumption begins when a preovulatory follicle receives LH signaling, resulting in a decrease in cyclic guanosine monophosphate (cGMP) and subsequent decline in cAMP leading to resumption [30]. Meiotic resumption may also occur when the oocyte is removed from the follicle, although in the horse meiotic resumption may be delayed for extended periods after removal [31]. Once meiosis has resumed the nuclear envelope will breakdown, generally referred to as germinal vesicle breakdown (GVBD). In addition to the dissolution of the nuclear envelope, GVBD is characterized by condensation of chromatin and formation of the meiotic spindle. Subsequently, the meiotic spindle coordinates segregation of the chromosomes and completion of the first meiotic division. Immediately after the completion of meiosis I the oocyte will proceed to metaphase II and arrest once more. The meiotic divisions of the oocyte are asymmetrical as to ensure that the oocyte retains critical cytoplasmic contents needed for embryo development [29]. Completion of meiosis I results in extrusion of a tiny

nonfunctional daughter cell, the first polar body, into the perivitelline space between the oocyte and zona pellucida [26]. The presence of the first polar body is therefore the standard marker of successful nuclear maturation.

Cytoplasmic Maturation 1.3.2

Concurrent to the events of nuclear maturation, the contents of the cytoplasm must be prepared for fertilization as to maximize developmental competence. Events of cytoplasmic maturation begin with GVBD and include the reorganization of vesicles, the golgi apparatus, and endoplasmic reticulum [32]. Among the most critical events is migration of cortical granules to the plasma membrane of the oocyte. At the time of fertilization, the cortical granules are exocytosed and their contents trigger hardening of the zona pellucida as a block to polyspermy, a process referred to as the cortical reaction [33-34]. Mitochondria must also relocate as part of proper cytoplasmic maturation. During maturation mitochondria are expected to migrate towards the central region of the oocyte. Mitochondria also arrange themselves around lipid droplets, providing easy access to a potential energy source during maturation. [35-36]. In equine oocytes, oocytes with heterogenous distribution of mitochondria throughout the ooplasm are considered more cytoplasmically mature as compared to those with a homogenous distribution [37-39]. These cytoplasmic changes are critical for both fertilization and subsequent embryo development. While these factors aren't easily evaluated in a clinical setting, they may be evaluated for research purposes as a measure of oocyte quality, or developmental competence.

Cumulus Expansion 1.3.3

In conjunction with oocyte maturation, the cumulus mass surrounding the oocyte undergoes a process referred to as expansion. This process is primarily characterized by cumulus cell deposition of a hyaluronic acid rich extracellular matrix. When hyaluronic acid interacts with

water it expands, resulting in expansion of the intercellular space between cumulus cells. As a result, the cumulus mass expands greatly in volume. This process is tied closely to development of the oocyte, which produces signals that promote the expansion of cumulus cells during maturation [40-41]. While expansion seems to be gonadotropin independent, the addition of FSH to culture media has been shown to enhance hyaluronic acid synthesis in mouse oocytes [42]. Cumulus expansion *in vitro* may be used as an indicator of oocyte quality where an expanded cumulus is generally an indicator of enhanced oocyte quality. Data from bovine oocytes has demonstrated that oocytes with expanded cumulus after IVM have higher rates of blastocyst development [43]. Similar data was presented in mice, with a positive correlation between cumulus expansion and rate of development to a 2-cell stage [34]. The factors that may affect cumulus expansion *in vitro* are numerous and not fully explored.

Metabolism of the Equine Cumulus Oocyte Complex 1.4

In addition to the transfer of signaling factors, the transzonal projections of the cumulus oocyte complex (COC) allow for transfer of nutrients necessary for oocyte development and survival [44-45]. Mice are the primary model by which COC metabolism has been explored where cumulus cells perform glycolysis to produce pyruvate for themselves and the oocyte [46]. Pyruvate enters the oocyte via transzonal projections where it is used for the citric acid cycle and oxidative phosphorylation to drive ATP production. The oocyte also may use other endogenous fuel sources for ATP production [47]. Similar metabolic pattern have been observed in porcine and bovine models as well [48-49]. On the other hand, metabolism of the equine COC is not well studied, and emerging data indicates that they may be metabolically distinct from other domestic species [11]. A recent study found that equine COC display a high level of oxidative

phosphorylation, yet most glucose consumed is accounted for by lactate production, rather than pyruvate, suggesting the use of an alternative fuel source for aerobic metabolism [10]. Proteomic and metabolomic analysis of cumulus cells found that high-glucose, low-lipid environments promote aerobic glycolysis while increasing lipid biogenesis, potentially to support continued fatty acid oxidation (FAO) [11]. Additionally, similar to porcine oocytes, equine oocytes are dark in appearance and thought to be lipid laden [50]. This growing metabolic profile suggests that equine oocytes may rely less on glycolysis derived pyruvate and more heavily on oxidation of endogenous lipid stores during maturation. While there is still much to be learned about equine oocyte metabolism, lipid metabolism may be a feasible target to enhance *in vitro* performance of oocytes.

Oocyte Retrieval 1.5

Postmortem Ovaries 1.5.1

In the case of a mare's unexpected death or necessary euthanasia, oocytes may be recovered from the ovaries for embryo production. Ovaries can be processed on site or transferred to a qualified facility for oocyte recovery. The industry standard for oocyte retrieval is to open individual follicles with a scalpel blade and scrape the entire surface of the follicle wall with a bone curette, yielding an average of 18 oocytes per mare [4]. While scraping remains the standard, more recent evidence suggests that ultrasound guided follicular aspiration may yield more oocytes per ovary as compared to scraping [51-52] Recovered oocytes are immature and may be held in commercial embryo-holding media overnight prior to IVM.

When ovaries cannot be processed on site, care must be taken to transport the ovaries as quickly as possible. Optimal outcomes are achieved when ovaries are received and processed

within 6 hours of the mare's death [4]. although some reports have shown similar results up to 10 hours after the mare's death [51]. During transport, ovaries should be allowed to cool to room temperature but should not be chilled below 12°C [53]. If transit time is expected to be less than 2 hours, ovaries may be shipped near body temperature (35-37°C); however, maintaining ovaries at body temperature for longer durations may result in detrimental effects on oocyte chromatin configuration [54].

Ovum Pick-Up 1.5.2

OPU, or TVA, is a minimally invasive procedure used to recover oocytes from the ovaries of live mares. OPU has grown in popularity as it allows for production of embryos by ICSI from subfertile mares, mares with busy competition schedules, and stallions with limited or poor quality semen [4, 55-56]. There are two primary approaches to OPU: aspiration of all immature follicles on the ovary to obtain numerous immature oocytes or aspiration of the dominant stimulated follicle (DSF) to obtain an *in vivo* matured oocyte [4].

Aspiration of immature follicles can be done regardless of stage of cycle, season, and presence of a dominant follicle. Typically, all visible follicles (≥5mm) are aspirated, provided the procedure is well tolerated by the mare. The COC is tightly adhered to the wall of immature follicles, necessitating repeated flushing (6-10 times) and scraping of the follicular wall with the needle to facilitate oocyte recovery [4]. Aspiration of immature follicles yields between 50%-70% recovery of oocytes [57-59]. After collection, immature oocytes remain arrested at the germinal vesicle stage and must undergo IVM before they can be used for *in vitro* embryo production. Notably, equine oocytes can be held overnight at room temperature without resumption of meiosis in the absence of meiotic inhibitory substances, with minimal impact on developmental competence [60-62]. The relative stability of immature equine oocytes, combined

with the ability to aspirate immature follicles at any time, provides considerable flexibility in timing of both OPU and subsequent *in vitro* procedures.

Alternatively, a ~35mm follicle during the estrus period may be stimulated with exogenous gonadotropins to induce final follicular maturation, resulting in a DSF. COC recovery rates from the DSF are ~80%, with the ease of recovery attributed to cumulus expansion in response to gonadotropin stimulation, which loosens COC attachment to the follicular wall [63-64]. In addition, the recovered oocyte has initiated maturation *in vivo*, and exhibits enhanced developmental competence as compared with immature oocytes [57-65]. While recovery is easier and the resulting oocytes show greater developmental competence, this approach requires precise timing and close coordination with an ICSI laboratory to be successful.

Despite the advantages of DSF aspiration, survey of practitioners performing OPU indicate the majority of aspirations involve immature follicles only [55]. This preference is likely driven by flexibility in timing, particularly for mares with demanding competition schedules. In addition, retrospective analyses of clinical programs have found that embryo production is directly correlated to the number of oocytes recovered [5, 66-67]. The monovular nature of the mare and inability to reliably induce superovulation support the strategy of aspirating all immature follicles to maximize oocyte yield and embryo production [1].

With continued advancement of *in vitro* techniques and oocyte collection methods, well-established OPU programs are reporting embryo production rates that exceed those achieved with conventional embryo flush. Mares are unlikely to produce more than one embryo per cycle with embryo flush, whereas some OPU-ICSI programs are reporting ~2 blastocysts produced per session [6, 9]. Although this average is impressive, there is considerable variability in embryo production between sessions, influenced by both mare and stallion related factors. One OPU-

ICSI program reported between 0-13 blastocysts produced per session, with at least one blastocyst produced in ~80% of sessions [5-6].

Owing to its flexibility and high embryo production potential, OPU has grown substantially in popularity. However, it is important to emphasized that OPU is not an innocuous procedure. Data collected from over 14,000 procedures indicates that major complications, most commonly peritonitis or ovarian abscess, occurred in 1 in 1000 procedures, and fatalities occurred 1 in 3000 procedures [55]. Although the overall risk is low, it is essential for clinicians and horse owners to carefully weigh the potential risk and benefits of these procedures.

Oocyte Holding and Transport 1.6

Following collection, whether from OPU or postmortem ovaries, oocytes must be transported to an ICSI laboratory. While some practices maintain in house laboratories, reducing logistical challenges associated with transport, most practices must ship or hold oocytes for extended periods of times before they can reach a laboratory. Conditions necessary to ship equine oocytes depend on the developmental stage and source of the oocyte.

In vivo matured oocytes, those that have been recovered from a DSF, present greater challenges for transport because they have resumed meiosis and are therefore more sensitive to environmental changes. Unlike immature oocytes, the *in vivo* matured oocyte must be maintained near body temperature (37.0-38.2°C) from the time of recovery. Exposure to 32°C for as little as 1.5 minutes has been shown to cause irreversible damage to the meiotic spindle [65]. To maintain appropriate temperature, these oocytes must be shipped in a portable incubator [4]. While insulated containers such as an Equitainer may be used, these systems tend to cool too rapidly, resulting in meiotic spindle damage and reduced blastocyst development compared with

portable incubator systems [65]. In addition, active metabolism of the DSF oocyte is also thought to require culture media as opposed to embryo holding media. An adequate volume of culture media must be provided as to prevent media acidification during transport [4].

In contrast, immature equine oocytes are uniquely resilient. Following collection, immature oocytes may be held in embryo holding media at room temperature (~20-25°C) for 24 hours or at ~15°C for two nights without detrimental effects on maturation rates or blastocyst development [31]. However, if immature oocytes are held above the optimal holding temperature, about 30°C or higher, resumption of meiosis may occur. Oocytes that resume meiosis under these conditions lack appropriate hormonal support in the embryo holding media, which is thought to contribute to abnormal maturation and reduced developmental competence [65]. Additionally, elevated holding temperatures are associated with alterations in chromatin configuration and meiotic competence, reducing success of *in vitro* procedures [60]. The resilience of immature oocytes allows greater flexibility in procedure scheduling and coordination with ICSI laboratories.

In Vitro Maturation 1.7

Following collection, either via OPU or postmortem ovaries, immature oocytes must be cultured *in vitro* to resume meiosis and reach a mature state. Oocyte maturation is primarily characterized by extrusion of the first polar body, indicating completion of meiosis I and arrest in metaphase II (MII). Because oocytes may not be fertilized until they have matured, IVM is essential to *in vitro* production of embryos [4]. Most established clinical programs report ~60% maturation rate for oocytes recovered by OPU [6-9]. In contrast, oocytes recovered from post-mortem ovaries typically exhibit lower maturation rates, between 30-50% [52, 68].

During IVM, oocytes are placed in maturation media that has been preequilibrated in an incubator and cultured for 24-36 hours. Historically, culture periods of 24-30 hours were standard; however, more recent evidence suggests that extended culture durations (30-36 hours) may be beneficial [9]. While Lazari et al. reported improved outcomes with prolonged culture, other studies have found no significant difference in maturation rates or subsequent developmental outcomes between standard and extended culture periods [8, 69]. Current evidence suggests that the optimal culture duration may depend on additional factors, particularly whether oocytes were held prior to culture and the length of holding time. Oocytes that have been held before culture appear to reach metaphase II more rapidly than those placed immediately into culture, which may require the full 30 hour maturation [69-70].

Despite it's critical importance, IVM in the horse remains less efficient than in other domestic species. Even under optimal conditions, maturation rates for oocytes from live mares rarely exceed 60%, and oocytes from post-mortem ovaries frequently exhibit substantially lower maturation rates [6-9, 52, 68]. Compounding this limitation, the developmental competence of *in vitro* matured equine oocytes is reduced as compared to *in vivo* matured oocytes. Blastocyst development rates are ~30% for *in vitro* matured oocytes, while *in vivo* matured demonstrate a 41-70% blastocyst rate. [57, 65]. Even when *in vitro* matured oocytes are transferred to a live mare's oviduct, providing an optimal physiologic environment for fertilization and embryo development, blastocyst rates remain lower than those achieved with *in vivo* matured oocytes [71]. Reduced developmental competence of IVM oocytes is likely attributable, at least in part, to incomplete cytoplasmic and nuclear maturation with cytoplasmic deficiencies increasingly recognized as a major limiting factor in the horse. [72-73]. While the factors contributing to poor developmental competence are likely multifactorial, suboptimal culture conditions may play a

significant role by failing to adequately support the metabolic demands of the maturing oocyte. Recent work has demonstrated that the protein and metabolite profile of cumulus cells was significantly modified in IVM oocytes as compared to *in vivo* matured oocytes. [11]. These metabolic alterations may help explain reduced developmental competence observed following IVM and highlight the need for continued optimization of equine oocyte culture conditions.

Several attempts have been made to optimize environmental conditions during IVM. Most labs perform IVM in a CO2 incubator in 5% CO2 and atmospheric oxygen tension (20-21%) [65]. In contrast, reduced oxygen tension (5-7%), is almost universally used for embryo culture across species due to the association between high oxygen tension and oxidative stress [74]. Although the exact oxygen tension of the equine ovarian follicle is unknown, humans follicular oxygen tension has been reported to be between 1-5.5% [75]. Although some equine ICSI laboratories utilized reduced oxygen tension during IVM [3], Limited equine-specific data exists regarding the benefits of reduced oxygen tension during IVM. A recent study of equine oocytes found no significant effect of reduced oxygen tension on metabolism during IVM [10]. However, studies in other species have demonstrated improved oocyte quality, metabolic changes, and enhanced oocyte morphology under reduced oxygen tension [76-77]. Further investigation is warranted to determine whether low oxygen tension is beneficial for equine oocyte maturation.

A wide variety of maturation media formulations are currently used for equine IVM, with most labs utilizing either tissue culture media 199 (TCM-199) with Earle's salts or Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) as the base media. A critical distinction between the two media is that TCM-199 contains a physiologic glucose concentration (5.6mM), whereas DMEM/F12 contains supraphysiologic glucose concentration (17-17.5mM). A recent

study found that supraphysiologic glucose during IVM lead to reduced non-mitochondrial respiration and decreased expression of lactate dehydrogenase in equine COC, suggesting alterations to glycolytic pathways [78]. Despite these metabolic differences, no significant differences in maturation rates have been observed between oocytes cultured in TCM199 and DMEM/F12 [79].

Regardless of the base media, maturation media are typically supplemented with various combinations of hormones, serum or follicular fluid, antibiotics, and other compounds. FSH is universally included, and LH is often added; however evidence from other species suggests that FSH alone may be sufficient to elicit effects comparable to LH *in vitro* [80]. E2 is also frequently included in maturation media, with studies in other mammalian species indicating that E2 supports nuclear maturation and cumulus expansion [81]. Epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) are included in some protocols. EGF has been shown to enhance maturation rates and cumulus expansion across multiple species [82-83], whereas IGF-1 appears to promote cytoplasmic maturation and enhance developmental competence in equine oocytes [84-85]. While fetal bovine serum is widely used in maturation media, preovulatory follicular fluid represents a promising alternative. Follicular fluid contains the endogenous milieu of factors present during *in vivo* maturation, including extracellular vesicles, which may provide more precise support of maturation *in vitro*. Use of follicular fluid in place of serum has been shown to enhanced cumulus expansion and modulated expression of MAPK and Wnt signaling pathways, which are important for normal maturation [86]. Additionally, recent work utilizing the follicular fluid secretome during IVM demonstrated improve maturation rates in postmortem derived equine oocytes [87]. Despite these advances, there remains no consensus regarding the

optimal composition of equine maturation media, with substantial variability in formulations used among laboratories [57, 65, 69, 88-89].

Although significant effort has been devoted to optimizing maturation media, considerable work remains to more effectively meet the metabolic requirements of the oocyte. A metabolomic analysis of preovulatory follicular fluid identified metabolites that are absent from commonly used maturation media. The identified metabolites were: acetylcarnitine, carnitine, citrate, creatine, creatine phosphate, fumarate, glucose-1-phosphate, histamine, and lactate [90]. Pyruvate was also reported to be absent from TCM199; however, pyruvate is routinely supplemented in maturation media due to its role in oocyte metabolism and antioxidant defense [91-92]. As detailed previously, a recent study of equine oocyte metabolism suggests that glucose is not the primary energy source during maturation and lipids may be a key source of energy during maturation [10, 11, 50]. If lipids are a key energy source during maturation it may be possible to target FAO as a potential means for improving IVM outcomes. Among the missing metabolites in maturation media are carnitine and acetylcarnitine. L-carnitine, the active form of carnitine and acetylcarnitine, is an essential co-factor for FAO that facilitates fatty acid transport into the mitochondrial matrix. Deficiency in L-carnitine is associated with reduced ability to metabolize lipids [93]. Given the potential reliance of equine oocytes on lipid metabolism, and the absence of carnitine in commonly used maturation media, supplementation with L-carnitine represents a biologically plausible strategy to enhance maturation, metabolic activity, and overall oocyte competence during IVM.

L-Carnitine 1.8

Metabolic Function 1.8.1

Carnitine is a small, polar compound present in all mammals that plays a central role in mitochondrial energy metabolism, particularly in cells reliant on FAO [94-95]. As detailed above, L-carnitine is an essential co-factor for FAO. L-carnitine facilitates the transport of long-chain fatty acids into the mitochondrial matrix allowing for their oxidation and subsequent production of cellular energy [95]. Activated long chain fatty acids, which are CoA esters of fatty acids, require L-carnitine to be transported to the mitochondrial matrix. Carnitine palmitoyltransferase-I (CPT-I) and -II (CPT-II) are located in the outer and inner mitochondrial membrane respectively and together mediate fatty acid transport as part of the carnitine shuttle. CPT-1 catalyzes the addition of L-carnitine to CoA esters to produce carnitine esters that are then transported to the intermembrane space of the mitochondria. CPT-II then mediates transport across the inner mitochondrial membrane while catalyzing the conversion of carnitine esters back to CoA esters that may begin FAO [94]. In addition to its essential role in FAO, L-carnitine supports pyruvate oxidation by maintaining an ideal ratio of acetyl-CoA/CoA supporting pyruvate dehydrogenase (PDH) function [93, 96]. CPT-II produces free carnitine in the mitochondrial matrix that may be converted to Acetylcarnitine by action of the enzyme carnitine acetyltransferase (CrAT). CrAT mediates the reaction of carnitine and Acetyl-CoA to produce acetylcarnitine and CoA. This reaction may also occur in the reverse allowing for production of free carnitine and Acetyl-CoA, maintaining an appropriate acetyl-CoA/CoA ratio to support pyruvate metabolism by PDH [94]. Furthermore, evidence supports that L-carnitine has an antioxidant effect *in vitro* by reactive oxygen species (ROS) scavenging and enhancing expression of antioxidant proteins [97-98].

In Vivo 1.8.2

In live animals, carnitine is primarily concentrated to skeletal and cardiac muscle [95]. Although they are capable of producing carnitine endogenously, it is primarily derived from the diet in most animals. Meat, fish, and dairy products are the primary source of carnitine , none of which the horse regularly consumes. As a result, the horse, and other herbivorous animals, rely heavily on carnitine biosynthesis to support their metabolic health [99]. Biosynthesis primarily occurs in the liver with the kidney and brain also showing capacity for biosynthesis. During biosynthesis, N^6-trimethyl-lysine (TML), a methylated lysine product released following lysosomal protein hydrolysis, serves as the first substrate of the biosynthetic pathway that yields butyrobetaine. Butyrobetaine is subsequently converted into carnitine [99]. While horses primarily rely on biosynthesis of carnitine, oral supplementation of L-carnitine may result in an increase in blood plasma concentration; however, the efficacy of absorption of L-carnitine is low with much of what is absorbed lost to renal excretion [100]. In obese mammals, excess lipids in circulation bind to free L-carnitine forming long-chain acylcarnitines that efflux from mitochondria and accumulate in body fluids. Resultant deficiency in L-carnitine is linked to incomplete FAO as well as impaired CrAT and PDH function [93, 101]. As reported by Catandi et al., in the obese mare dietary supplementation with L-carnitine is sufficient to elevate total carnitine levels supporting metabolic function [102]. In short, biosynthesis of L-carnitine in the horse is sufficient to support normal metabolic function; however, in the obese animal L-carnitine supplementation may be beneficial to support normal cellular metabolic function. While systemic carnitine levels appear sufficient in healthy animals to support normal metabolism *in vivo*, absence of carnitine from IVM media may represent a critical discrepancy between the physiologic follicular environment and current culture systems.

In Vitro 1.8.3

Across a number of species L-carnitine has been explored as a potential enhancer of IVM. In bovine models, supplementation between 0.3-0.6 mg/mL L-carnitine significantly enhanced nuclear maturation rates [103]. Knitlova et al. reported that nuclear maturation in oocytes from small follicles, generally deemed less competent, tended to be enhanced along with significantly increased fertilization rates [104]. Not all studies reported enhanced maturation with L-carnitine treatment, however, they did report reduction in oocyte lipid content. The reduction in lipid content may result from enhanced lipid metabolism in response to supplemented L-carnitine [105]. Beyond maturation, assessment of embryo development revealed improved blastocyst formation rates, indicating enhanced developmental competence of oocytes with L-carnitine treatment [103, 106]. Studies of porcine oocytes reveal much the same, with 0.5-0.6 mg/mL L-carnitine supplementation enhancing nuclear maturation [107, 108]. Treatment resulted in redistribution of mitochondria to the center of the oocyte accompanied by significant reduction in lipid droplets. Additionally, levels of glutathione, a potent antioxidant, was increased concomitant to a reduction in ROS [107]. Cleavage and blastocyst rates were enhanced by L-carnitine, although Somfai et al. reported reduced blastocyst rate likely associated with high polyspermy rates in their culture system [107, 108]. Finally, buffalo oocytes treated with 0.5mg/mL L-carnitine display increased cumulus cell expansion with a significant improvement to maturation and blastocyst rates of "fair" quality oocytes so that they were comparable to "good" quality oocytes [109]. Together, evidence across species supports that L-carnitine supplementation may enhance oocyte maturation, reduce oxidative stress, support mitochondrial function, and increase cumulus expansion resulting in more developmentally competent oocytes. Most commonly, L-carnitine is supplemented at concentrations between 2-4 mM where the greatest biological effect is observed [103, 105-109]. While these concentrations exceed what is

seen in equine preovulatory follicular fluid, 0.37 mM acetylcarnitine and 0.09 mM carnitine, higher concentrations are often necessary to account for the static nature of *in vitro* culture systems [90]. Use of concentrations higher than 4mM L-carnitine have shown at best a negation of the beneficial effects of L-carnitine and at worst a reduction in nuclear maturation and blastocyst rates [103, 107, 108]. Despite the strong body of evidence across species, the effects of L-carnitine supplementation during equine IVM remain entirely unexplored.

Summary 1.9

Despite significant efforts, IVM remains suboptimal in the horse, largely attributed to culture conditions that fail to properly support the oocyte. Given the increasing commercial importance of OPU-ICSI, it is crucial to reassess and enhance current maturation media. The unique metabolism of the oocyte is largely unexplored making it difficult to properly optimize maturation media; however, evidence indicates that the equine COC may utilize lipids as a significant energy source during maturation. L-carnitine appears to play a role in supporting COC metabolism *in vivo*, but is not included in commonly used maturation media. The addition of L-carnitine to maturation media may better support the oocyte metabolism potentially enhancing oocyte maturation, quality, and alter metabolism *in vitro*.

CHAPTER 2: MATERIALS AND METHODS

Research hypothesis 2.1

This study hypothesizes that supplementing IVM media with L-carnitine will improve both the maturation rates and quality of *in vitro* matured equine oocytes. We further hypothesize that L-carnitine supplementation will alter the metabolic profile of *in vitro* matured cumulus oocyte complexes. If L-carnitine supplementation enhances maturation and oocyte quality, these findings could contribute to enhanced *in vitro* production of equine embryos and provide new insights into unique metabolism of the equine oocyte.

Facilities 2.2

All animal work was performed at Colorado State University Equine Reproduction Laboratory (ERL). The ERL is an equine breeding facility that can house over 300 horses on over 70 acres of land. There are more than 100 stalls and 40 acres of land devoted to group mare housing in dry lot paddocks. There are dedicated stocks for routine mare reproductive evaluations and standing surgical procedures.

Hypothesis 1 2.3

L-carnitine supplementation during IVM of equine oocytes will increase maturation rates and quality of *in vitro* matured oocytes.

Mare Management 2.3.1

Between the months of July-September, 20 grade mares (15-20 years of age) were maintained on dry lots at the ERL. During this time, mare reproductive cycles were regularly

monitored by transrectal ultrasonography. When a dominant follicle ≥ 35 mm in diameter was observed in concert with edema consistent with estrus, ovulation induction agents were given. Human chorionic gonadotropin (1500 IU, intravenous; Chorulon, Merck Animal Health, Madison, NJ) and histrelin in aqueous base (0.5mg, IM; Doc Lane, Lexington, KY) were administered to induce ovulation. 21.2 +/- 1.8 hours after ovulation induction, dominant stimulated follicle and all immature follicles ≥ 5 mm present on both ovaries were aspirated by TVA.

Follicle Aspiration 2.3.2

Follicle aspirations were performed by experienced veterinarians. Prior to entering the aspiration stocks, mares received xylazine (100mg, IV; Rompun, Dechra Veterinary Products, Overland Park, KS) for sedation. At this time, using sterile gloves, a urinary catheter was placed. The mare then received detomidine hydrochloride (10mg, IV; Dormosedan, Zoetis, Parsippany, NJ), butorphanol tartrate (5mg, IV; Torbugesic, Zoetis, Parsippany, NJ), and N-butylscopolammonium bromide (140mg, IV; Buscopan, Boehringer Ingelheim, Ingelheim, Germany) to sedate the mare and relax rectal tone, limiting risk for rectal irritation/tear.

Follicles were visualized with a specialized TVA ultrasound probe, which contains a needle guide to facilitate the passage of a 12 gauge double lumen needle. The TVA probe was placed against the cranial vaginal wall on either side of the cervix while, per rectum, the ovary was drawn against the vaginal wall to facilitate visualization and subsequent aspiration of follicles. If the dominant stimulated follicle (DSF) was still present at the time of aspiration, it was aspirated first to facilitate the collection of pure follicular fluid. For the DSF, pump pressure was set to ~150mmHg. The first ~10mL of follicular fluid was collected in a 50mL conical tube before switching to a 250mL collection bottle. Once the follicle had completely deflated, the

follicle was flushed three times with commercial embryo flush media (ABT Complete Flush, ABT 360, Pullman, WA) with 10 IU/mL heparin sodium (McKesson, Irving, TX) warmed to body temperature. When aspiration of the DSF was completed, pump pressure was reduced to ~130mmHg and a 500mL collection bottle was used for the remainder of the aspiration. All immature follicles ≥ 5 mm were aspirated on each ovary. Each follicle was flushed ~10 times with embryo flush media while the operator scrapped the follicular wall to facilitate dislodgment of the COC.

Following aspiration, the mare received flunixin meglumine (500mg, IV; Banamine, Merck, Rahway, NJ) for analgesia and ceftiofur crystalline free acid (Excede®, Zoetis, Parsippany, NJ) when an antimicrobial was deemed necessary. All mares were subjected to rectal temperature checks for 72 hours following the aspiration and monitored for signs associated with abdominal discomfort (anorexia or other clinical signs consistent with colic). Each mare had a rest period of at least 2 weeks between procedures in which no TVA procedures were performed.

Cumulus Oocyte Complex Isolation 2.3.3

Immediately after the aspiration, the aspirate was searched for collected COC. DSF aspirate was searched first, lending to its particularly sensitive nature. A stereoscope fitted with a heated stage at 38.2°C was used to search unfiltered aspirate in large cell culture dishes. The COC were isolated using a ¼ cc straw and rinsed gently in holding media (TCM199 with Hank's salts (Thermo Fisher Scientific, Waltham, MA) + 10% FCS (HyClone™ Characterized Fetal bovine Serum, Cytiva, Marlborough, MA) + 50 µg/mL gentamycin (Sigma Aldrich, Saint Louis, MO) + 0.2mM sodium pyruvate (Sigma Aldrich, Saint Louis, MO)) at 38.2°C. The rinsed COC were then transferred to 2.5 mL of culture media (TCM199 with Earle's salts (Thermo Fisher

Scientific, Waltham, MA) + 10% FCS + 50 µg/mL gentamycin + 0.2mM sodium pyruvate) that has been preequilibrated overnight at 38.2°C in 8.5% CO2 and air.

Immature follicle aspirate was filtered using an embryo filter cup (EZ Way Filter, spi-mfg, Canton, TX) rinsed with fresh flush media until aspirate was clear. COC were then isolated from the embryo filter and rinsed in holding media at room temperature. Once all COC were isolated, they were moved to a 5 mL culture tube with ~4mL of holding media at room temperature. Immature oocytes were held at room temperature overnight (17-23 hours).

Oocyte Culture and Denudation 2.3.4

The oocyte collected from the DSF were be cultured at 38.2°C in 8.5% CO2 and air until ~40 hours after the time of ovulation induction to ensure proper time to maturation. After the holding period, immature COC were transferred to individual 30µL microdroplets of maturation media with an oil overlay (OvOil™, Vitrolife, Englewood, CO) that had been preequilibrated overnight at 38.2°C in 8.5% CO2 and air. Each treatment was prepared in its own 35mm dish with wash droplets, culture droplets, and media only droplets. Oocytes were randomly distributed between three treatment groups: standard maturation media (TCM199 with Earle's salts + 10% FCS + 50 µg/mL gentamycin + 0.2mM pyruvate + 5mU/mL FSH (Sigma Aldrich, Saint Louis, MO)), 2mM L-carnitine supplemented (standard maturation media + 2mM L-carnitine hydrochloride (CO283, Sigma Aldrich, Saint Louis, MO)), and 4mM L-carnitine supplemented media (standard maturation media + 4mM L-carnitine hydrochloride). Immature oocytes were then cultured for 33 +/- 1.3 hours at 38.2°C in 8.5% CO2 and air.

At the end of the culture period oocytes were denuded of cumulus cells by sequential pipetting with a Stripper™ Pipettor (CooperSurgical, Trumbull, CT) in G-MOPS (Vitrolife, Englewood, CO) + 0.4% BSA (Sigma-Aldrich, St. Louis, MO) with 80 IU/mL hyaluronidase

(Sigma-Aldrich, St Louis, MO). A 200 µm stripper tip was used initially and if needed a 135 µm stripper tip was used to completely denude the oocyte.

Evaluation of Maturation and Cumulus Expansion 2.3.5

Immediately following the culture period, prior to denudation, the cumulus cells were classified as expanded or compact based on visual inspection under a stereoscope. Following denudation the oocytes were evaluated as immature (no polar body present), mature (polar body present), or degenerated.

Oocyte Staining 2.3.6

Prior to the end of the culture period, a working solution consisting of 1µg/mL Hoechst 33342, 1 µM H2DCFDA, and 50 nM MitoTracker Deep Red was prepared in G-MOPS™ PLUS (Vitrolife, Englewood, CO). 500µL of staining solution was placed in each well of a 4 well dish and held at 38.2°C. Denuded mature oocytes were rinsed in three 100µL droplets of staining solution before being moved to the 4 well dish with staining solution. Oocytes were incubated for 30 minutes at 38.2°C.

Following incubation with the stain, oocytes were washed in three 100µL droplets of G-MOPS™ PLUS and moved to a 35 mm glass bottom dish in a 100µL droplet of the same media for imaging. Stained oocytes were immediately examined for fluorescence intensity using an inverted Revolve hybrid microscope (Echo, San Diego, CA) at excitation/emission 380/450 for Hoechst 33342, 470/525 for H2DCFDA, and 630/700 for Mitotracker Deep Red, using fixed gain and exposure settings. Gray scale pictures acquired at 100x magnification and assessed for average pixel intensity within individual oocytes using ImageJ software. Mitochondrial

distribution was quantified as the coefficient of variation (CV) of Mitotracker deep red fluorescence intensity.

Statistical Analysis 2.3.7

Normality of fluorescence data was assessed using the Shapiro-Wilk test. Multiple comparisons were made using a one-way ANOVA followed by Tukey's HSD or the Kruskal-Wallis test followed by Dunn's test where appropriate. Significance of oocyte maturation and cumulus expansion rates were analyzed using a chi-square test of independence. Statistical significance was defined as $p<0.05$.

Hypothesis 2 2.4

L-carnitine supplementation during IVM of equine oocytes will alter metabolism of *in vitro* matured cumulus oocyte complexes.

Spent Media Analysis 2.4.1

Following the culture period, 20µL of spent media and 20µL from media only droplets were collected in microcentrifuge tubes. Media was stored in a -80°C freezer until the time of analysis. Media samples were submitted to the CSU analytical resource core (ARC) for metabolite quantification. Samples consisted of 7 media only samples from each treatment and 7 spent media samples from each treatment for a total of 42 samples. Spent media samples were not restricted to those that contained a mature oocyte.

Sample Preparation 2.4.2

Leucine-D10 was purchased form Cambridge isotypes. Succinic acid-13C4 was purchased from Sigma-Aldrich. Solvents and reagents mentioned elsewhere were purchased from Fischersci.

Media samples (18µL) were mixed with 10µL of internal standard solution containing 83 µg/mL of leucine-D10, 83 µg/mL trehalose, and 42 µg/mL of succinic acid-13C4 in 50% methanol/water. Ice-cold methanol (92µL) was added, and the samples were briefly vortexed, incubated at -20°C overnight, followed by centrifugation at 15,000 g for 10 min at 4°C. The supernatant (100µL) was recovered, dried down, resuspended in 50µL of pyridine containing 25 mg/mL of methoxyamine hydrochloride, incubated at 60°C for 45 min, vigorously vortexed for 30 s, sonicated for 10 min, and incubated for an additional 45 min at 60°C. Next, 50µL of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS, Thermo Scientific) were added, and samples were vigorously vortexed for 30 s, incubated at 60 °C for 30 min. Then 10µL of samples were pooled to generate a QC sample, which was then split into multiple vials.

GC-MS Analysis 2.4.3

Gas chromatography-mass spectrometry (GC-MS) was used to identify relative quantity of metabolites in media. Metabolites were detected using a Trace 1310 GC coupled to a Thermo ISQ mass spectrometer. Samples (1µL) were injected at a 15:1 split ratio to a 30 m TG-5MS column (Thermo Scientific, 0.25 mm i.d., 0.25 µm film thickness) with a 1.2 mL/min helium gas flow rate. GC inlet was held at 285°C. The oven program starts at 80°C for 30 s, followed by a ramp of 15°C/min to 330°C, and an 8 min hold. Masses between 50-650 m/z are scanned at 5 scans/sec under electron impact ionization. Transfer line and ion source are held at 300 and

260°C, respectively. Pooled QC samples were injected after every 6 study samples. The injection order of samples was again randomized.

Data Processing 2.4.6

GC-MS data was processed using Chromeleon 7.2.10 software (Thermo Scientific). The quantification/confirming ions and GC retention time for each acid can be found in Table 1. Peak areas were extracted for target compounds detected in biological samples and normalized to the peak area of the appropriate internal standard or surrogate in each sample.

Table 1. GC-MS data processing method

	RT (min)	Quantifier m/z	Qualifier m/z
Pyruvate-1MEOX-1TMS	3.799	174.1	115.1
Lactate-2TMS	3.904	191.1	147.1
alanine-2TMS	4.292	116.12	147.1
valine-2tms	5.354	144.1	218.1
Leucine-D10	5.816	168.2	242.25
leucine-2tms	5.866	158.15	218.1
isoleucine-2tms	6.071	158.15	218.1
proline-2tms	6.122	142.1	216.1
succinate-13C4-2tms	6.201	251	176
glycine-3tms	6.204	174.12	248.14
serine-3tms	6.662	204.1	218.1
threonine-3tms	6.911	218.1	291.1
aspartate-3tms	8.02	232	218.1
methionine-2tms	8.024	176.1	293
oxoproline-2tms(glutamate)	8.069	156.1	230.1
cysteine	8.33	220.1	218.1
glutamate-3tms	8.802	246.1	128.1
phenylalanine-2tms	8.903	218.1	192.1
asparagine-3tms	9.23	116.1	231.1
glutamine-3tms	9.961	156.1	245.15

ornithine-4tms(arginine-NH3)	10.319	157.1	256.2
glucose1	10.96	319.1	205.1
lysine-4tms	11.007	174.1	317.22
glucose2	11.09	319.1	205.1
tyrosine-3tms	11.135	218.1	280.2
glucose3	11.235	319.1	205.1
tryptophane-3tms	12.934	202.11	291.15
trehalose	15.85	361.1	191.1

Statistical Analysis 2.4.4

Normality of data was assessed using the Shapiro-Wilk test. Multiple comparisons were made using a one-way ANOVA or Kruskal-Wallis test where appropriate. Pairwise comparisons were made using Tukey's HSD or Dunn's test where appropriate. Simple linear regression was performed to establish relationships between metabolites. Statistical significance was defined as a $p<0.05$.

CHAPTER 3: RESULTS

Cumulus expansion Rates 3.1

COC were evaluated following the maturation period and determined to either have a compact or expanded cumulus mass (Table 3) Cumulus expansion was significantly greater ($P<0.0001$) in L-carnitine treated groups (73.4% and 67.1% for 2mM and 4mM LC treatments respectively) as compared to standard maturation media (33.3%) (Figure 2).

Table 3. Cumulus Expansion

Group	No. Oocytes in Culture	No. Expanded (%)	No. Compact (%)
Standard IVM	81	27 (33.3)	54 (66.6)
2mM LC	79	58 (73.4)	21 (26.6)
4mM LC	79	53 (67.1)	26 (32.9)

Oocyte Maturation Rates 3.2

In total, 42 OPU procedures were performed in which 465 immature follicles were aspirated yielding 254 oocytes (6.2 +/- 3.7 oocytes per aspiration), not including those deemed degenerated or inviable at the time of collection. Collected oocytes were split evenly among treatment groups. Oocytes were deemed mature, immature, or degenerated after culture (Table 2). The proportion of oocytes that reached maturity (61.7%, 67.1%, and 60.8% for standard, 2mM, and 4mM groups respectively) was not significantly different ($p = 0.982$) across treatments (Figure 1).

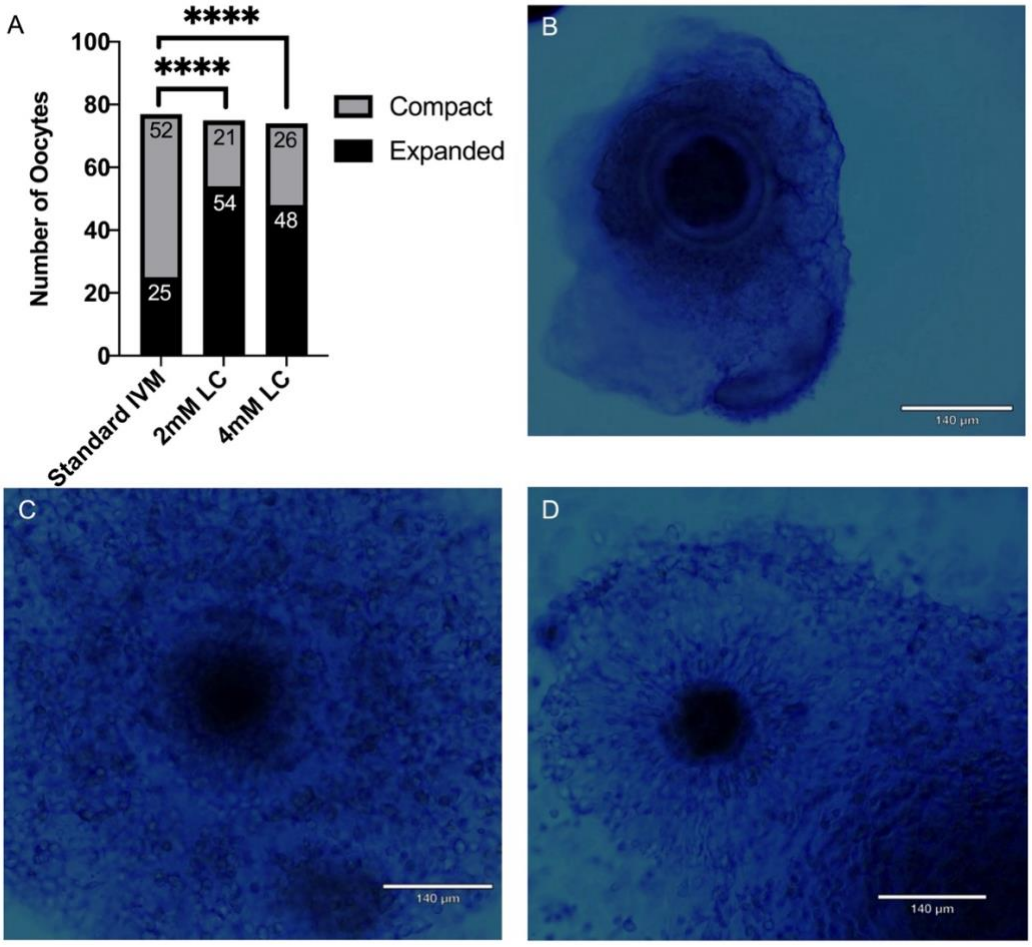

Figure 1. Cumulus expansion was significantly enhanced by L-carnitine supplementation.(A) Statistical significance was evaluated using the chi-square test for independence followed by Fisher's exact test for pairwise comparison. (B) Immature oocyte before IVM. (C) Oocyte with non-expanded cumulus after maturation. (D) Oocyte with expanded cumulus after maturation.

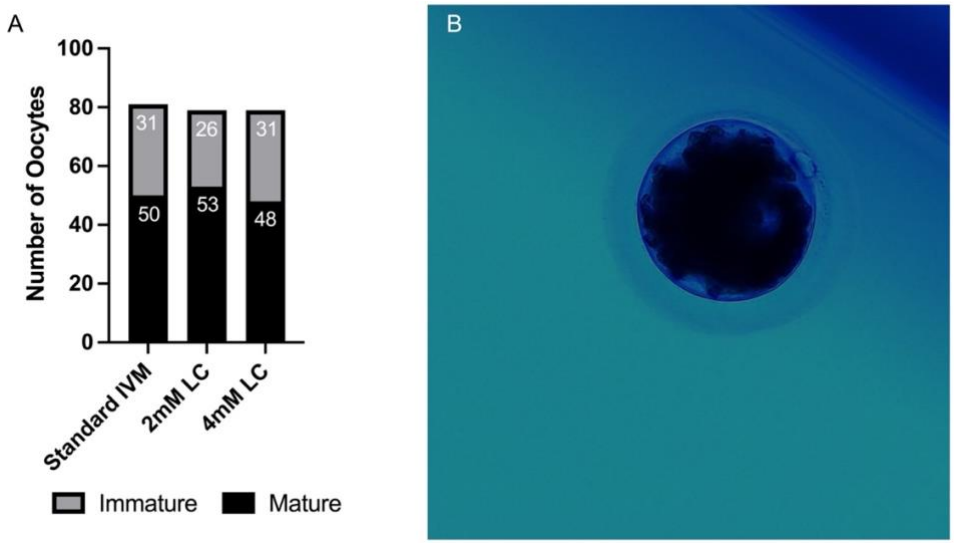

Figure 2. Oocyte maturation was unaffected by L-carnitine supplementation. (A) No statistical difference (P=0.6733) was found between maturation rates of each treatment. Statistical significance was evaluated using the Chi-square test of independence. (B) Image of a mature denuded oocyte.

Table 2. Oocyte Maturation

Group	No. Oocytes in Culture	No. Mature (%)	No. Immature (%)	No. Degenerated (%)
Standard IVM	81	50 (61.7)	22 (27.2)	9 (11.1)
2mM LC	79	53 (67.1)	21 (26.6)	5 (6.3)
4mM LC	79	48 (60.8)	23 (29.1)	8 (10.1)

Reactive Oxygen Species Levels 3.3

ROS levels in individual mature oocytes were quantified by fluorescence intensity of H2DCFDA. There was no significant difference (P>0.9999) in ROS levels between all treatment groups (Figure 3).

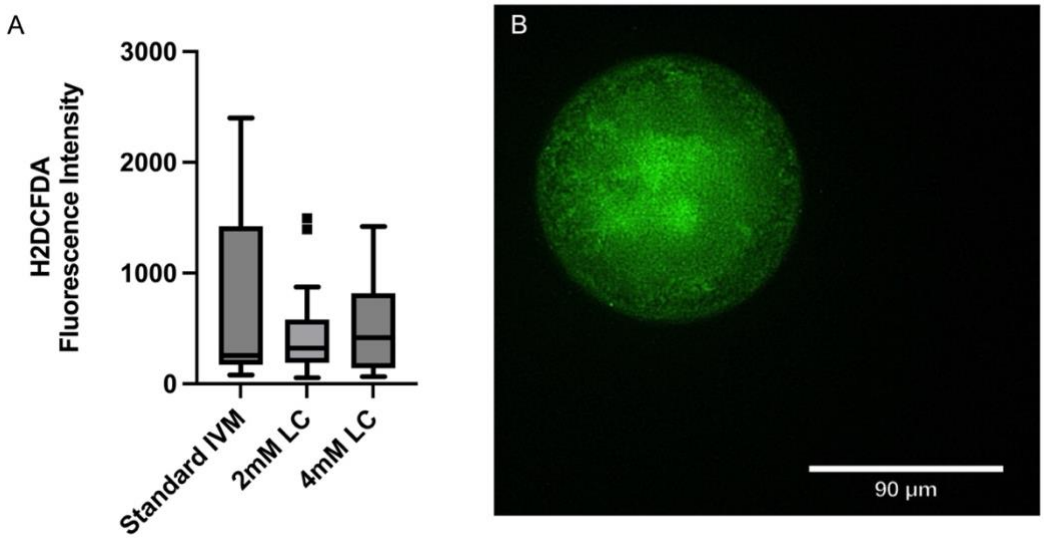

Figure 3. ROS levels were unchanged by supplementation with L-carnitine. (A) Statistical significance was evaluated using the Kruskal-Wallis test. There was no difference (P>0.9999) in H2DCFDA fluorescence intensity between all treatments (B) Oocyte stained with H2DCFDA.

Mitochondrial Function and Distribution 3.4

Mitochondrial membrane potential, quantified by fluorescence intensity of MitoTracker Deep Red stain, was significantly reduced (p=0.0374) in the 4mM LC treatment group as

compared to standard IVM. Mitochondrial function did not differ (P>0.05) between standard IVM and 2mM LC or 2mM LC and 4mM LC treatments (Figure 4).

Mitochondrial distribution, evaluated by the CV of MitoTracker Deep Red fluorescence intensity, did not differ significantly (P>0.05). across treatment groups (Figure 4).

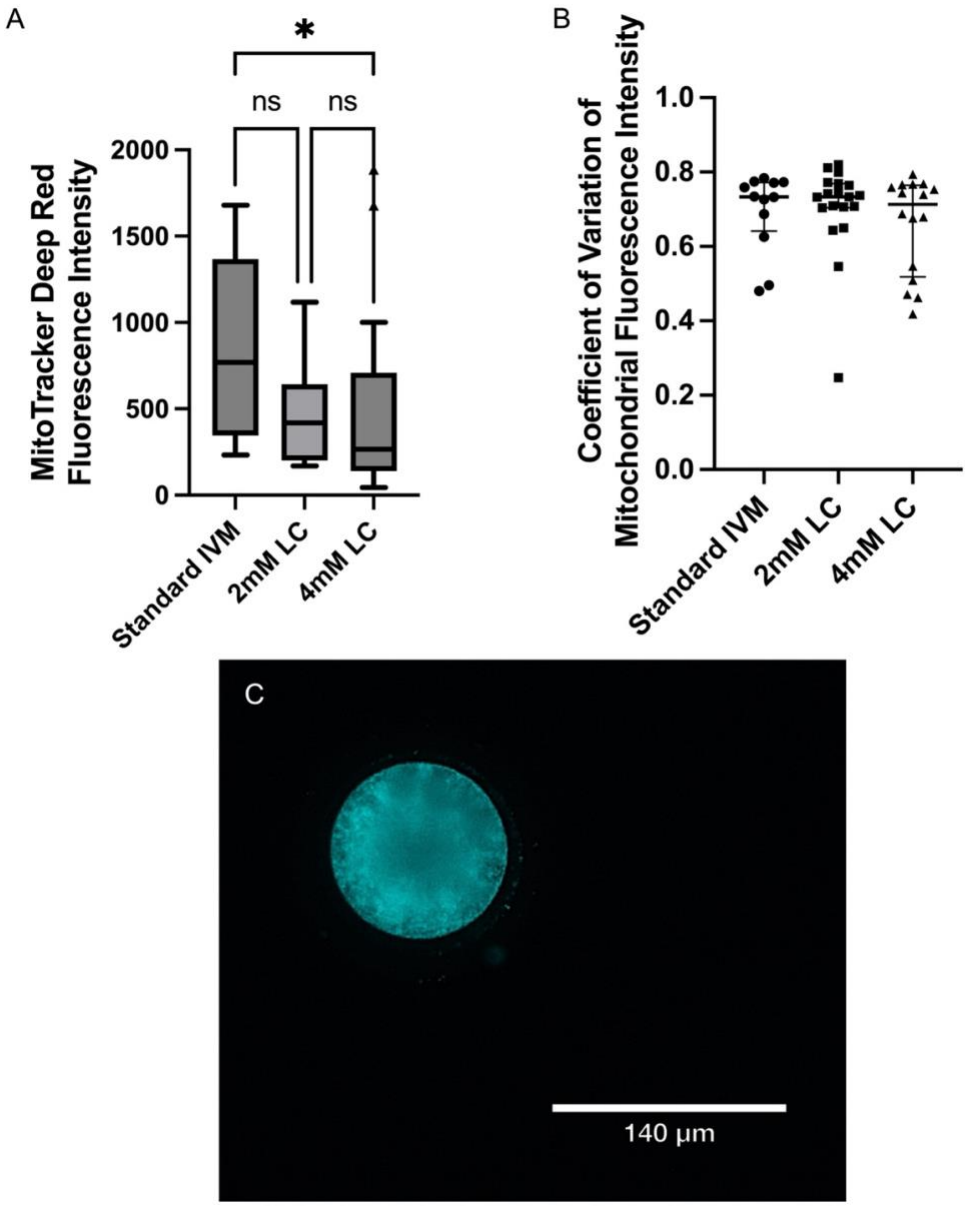

Figure 4. Oocyte mitochondrial function is reduced by 4mM L-carnitine treatment. Statistical significance was evaluated using the Kruskal Wallis test followed by Dunn's test for pairwise comparison. (A) Mitotracker deep red fluorescence intensity was significantly reduced (p=0.0374) between standard and 4mM LC treatment. There was no significant difference in fluorescence intensity between standard and 2mM LC (p=0.1730) or 2mM LC and 4mM LC (p>0.9999). (B) Coefficient of variation of mitochondrial deep red fluorescence intensity was not significantly different (P>0.9999) across all treatments. (C) oocyte stained with Mitotracker deep red.

Spent Media Analysis 3.5

Media only and spent media droplets were analyzed by GC-MS, quantifying relative quantity of glucose, pyruvate, lactate, and amino acids in media (Figure 5).

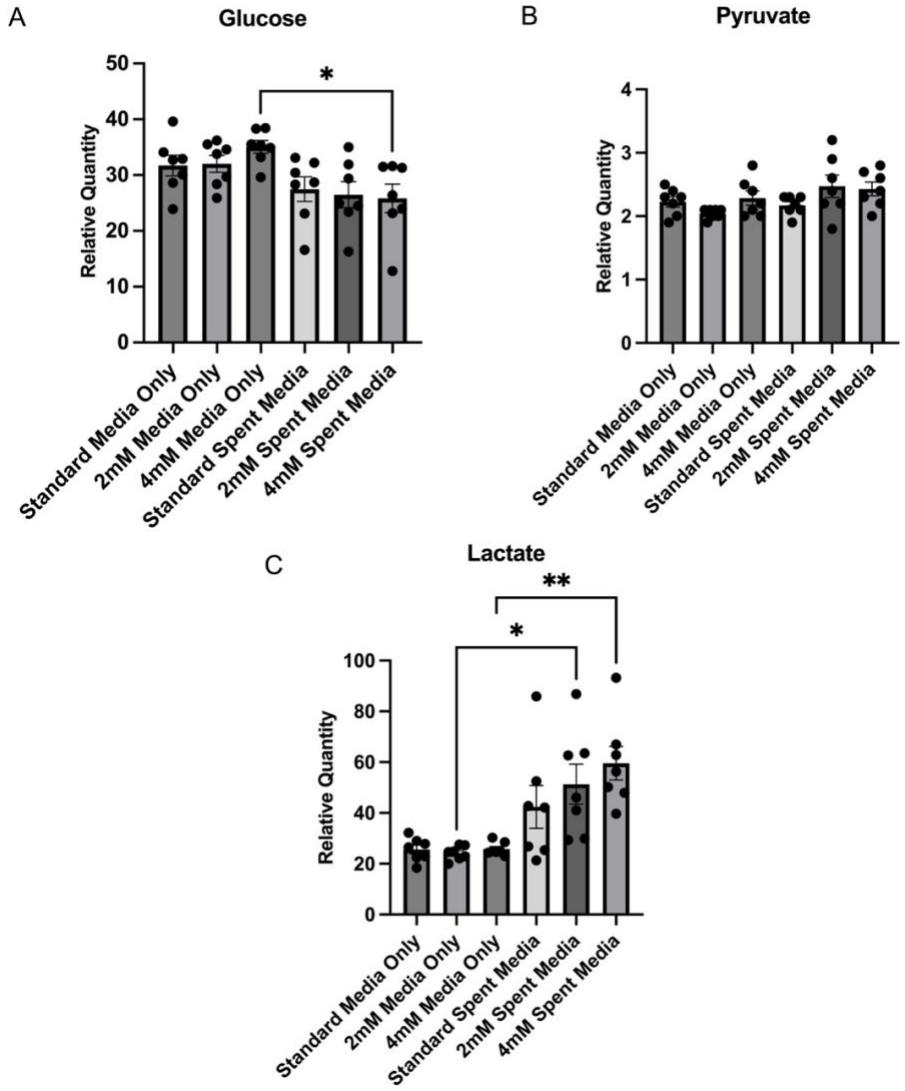

Figure 5. Glucose consumption and lactate production were altered in L-carnitine treated media. All data is presented as mean +/- SEM. Shapiro-Wilk test was used to assess normality of data followed by either one-way ANOVA or Kruskal-Wallis test where appropriate. Pairwise comparisons were made with either Tukey's HSD or Dunn's test. (A) There was no significant difference in glucose concentration between spent media of all treatment groups. There was a significant decrease ($p=0.0267$) in glucose in 4mM spent media as compared to media only droplets. Glucose did not differ between media only and spent media from either the standard or 2mM treatment groups ($p=0.6699$ and $p=0.3841$ respectively). (B) Pyruvate did not differ significantly ($p>0.05$) between media only and spent media or between treatments. (C) There was no significant difference in lactate between spent media of all treatment groups. There was a significant increase in spent media lactate relative to media only droplets in the 2mM and 4mM treatment ($p=0.0152$ and $p=0.0014$ respectively). Lactate did not differ between media only and spent media of the standard treatment ($p=0.2842$).

There was no significant difference ($p<0.05$) in concentration of amino acids between media only droplets and spent media or between treatment groups. When comparing media only and spent media droplets glucose was significantly decreased ($p=0.0267$) in spent media of the 4mM group, while standard ($p=0.6699$) and 2mM ($p=0.3841$) treatments showed no statistical difference. Glucose did not differ ($p>0.05$) in spent media of the different treatments. No significant difference ($p>0.05$) was observed in pyruvate between media only and spent media droplets or between treatments. In both the 2mM and 4mM treatment lactate was significantly increased ($p=0.0152$ and $p=0.0014$ respectively) in spent media droplets as compared to media only, while lactate did not differ in the standard treatment ($p=0.2842$). Lactate did not differ significantly ($P>0.05$) in spent media between treatments.

Glucose and lactate showed strong linear correlation (Figure 6) in spent media across all treatments (standard: $R^2=0.8435$ $p=0.0035$; 2mM: $R^2=0.7468$ $p=0.0121$; 4mM $R^2=0.8518$ $p=0.0030$). Conversely, there was no evidence of a linear correlation (Figure 6) between pyruvate and glucose in spent media of all treatments (standard: $R^2=0.3322$ $p=0.1756$; 2mM: $R^2=0.06694$ $p=0.3587$; 4mM $R^2=0.09062$ $p=0.5118$).

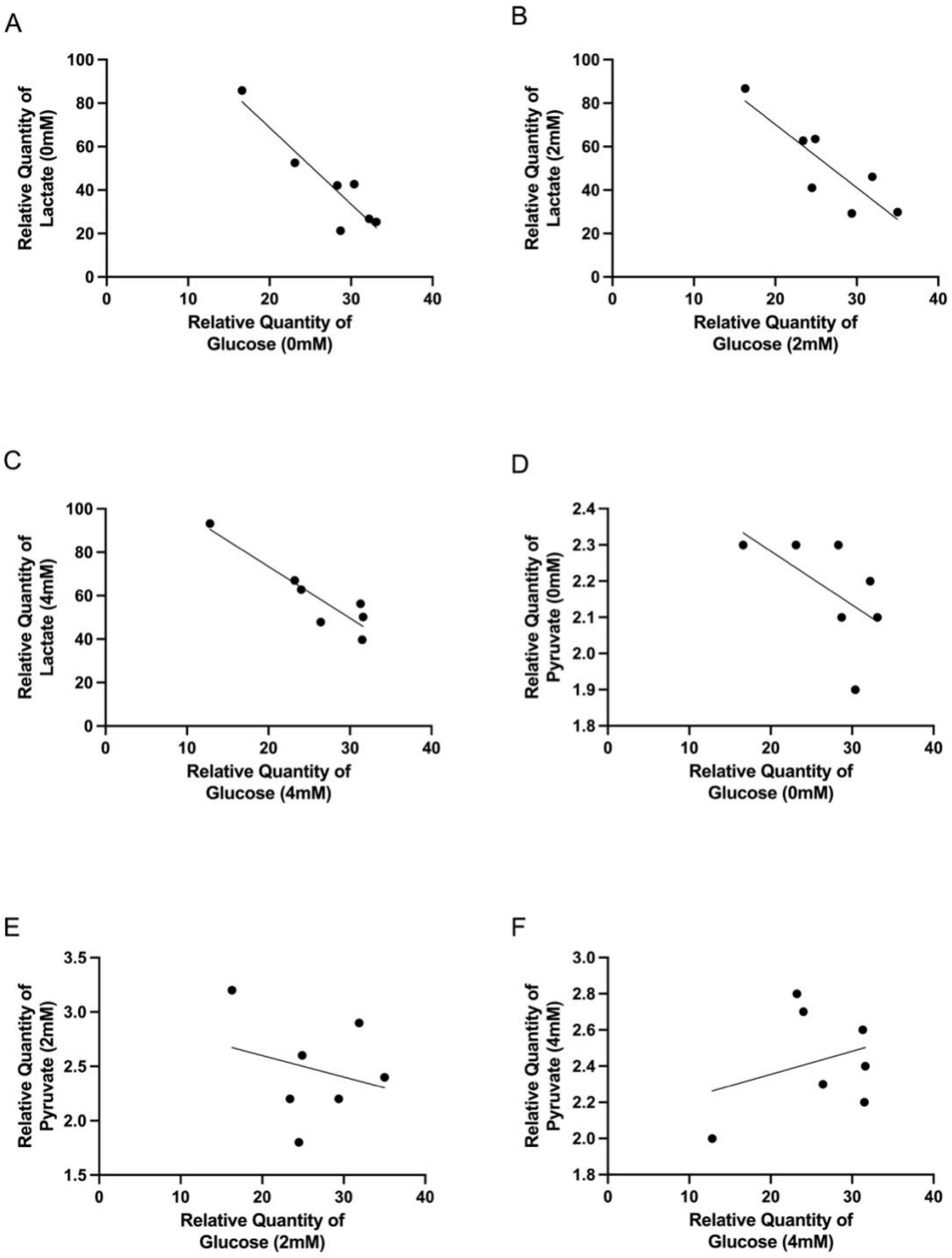

Figure 6 Consumption of glucose and production of lactate strongly correlate. Simple linear regression analysis was used to evaluate the relationship between glucose and lactate and glucose and pyruvate. (A) Lactate and glucose are correlated in the standard treatment (R^2=0.8435 p=0.0035). (B) Lactate and glucose are correlated in the 2mM treatment (R^2=0.7468 p=0.0121). (C) Lactate and glucose are correlated in the 4mM treatment (R^2=0.8518 p=0.0030) (D) Pyruvate and glucose are not correlated in the standard treatment (R^2=0.3322 p=0.1756). (E) Pyruvate and Glucose are not correlated in the 2mM treatment (R^2=0.06694 p=0.3587). (F) Pyruvate and Glucose are not correlated in the 4mM treatment (R^2=0.09062 p=0.5118).

CHAPTER 4: DISCUSSION

Oocyte nuclear maturation did not differ between treatments 4.1

Nuclear maturation rates did not differ significantly between all treatment groups. This is contrary to reports in other species where L-carnitine supplementation enhanced nuclear maturation rates [103, 107-109]; however, not all reports indicated an effect of L-carnitine on nuclear maturation. Knitlova et al. reported enhanced, but not statistically significant, maturation in developmentally less competent bovine oocytes [104]. Meanwhile, Zhao et al. reported no differences in bovine oocyte maturation following treatment with L-carnitine [105]. As such, the lack of change in nuclear maturation is not entirely surprising within our experiment; however, other factors may have contributed to this result. In particular, no system was utilized to evaluate or grade immature oocytes before dividing them among treatment groups. While no standardized system exists for grading of immature equine oocytes, visual evaluation of density and amount of cumulus cells, as described by Bertero et al. [110], may have allowed for grouping of oocytes of similar quality allowing for more in depth analysis of L-carnitine's impact. While the addition of L-carnitine did not increase nuclear maturation rates, it also did not decrease maturation rates indicating that L-carnitine does not negatively impact maturation.

Cumulus expansion was enhanced by supplementation with L-carnitine 4.2

The rate of cumulus expansion was found to be significantly increased in both L-carnitine treated groups as compared to the standard maturation media. This reflects observations seen by Modak et al. in which buffalo COC showed enhanced cumulus expansion with L-carnitine supplementation [109]. Generally speaking, the expansion of the cumulus mass is associated

with an increase in oocyte developmental competence [43]. The significant increase in cumulus expansion following L-carnitine treatment may indicate enhanced developmental competence of oocytes; however, true assessment of developmental competence would require fertilization and evaluation of blastocyst formation rate. The rise in cumulus expansion may be associated with metabolic alterations in response to L-carnitine treatment. The bidirectional signaling between the oocyte and cumulus cells is critical for normal and complete maturation, producing developmentally competent oocytes [25-26]. Specifically, the cumulus cells produce metabolites and signaling molecules essential for normal maturation. In turn, the oocyte produces signaling molecules to alter cumulus function, ultimately promoting cumulus expansion [40-41,47]. While we cannot pinpoint a specific cause, it may be possible that L-carnitine supplementation supported cumulus or oocyte metabolism permitting an enhanced rate of cumulus expansion. Regardless, the expansion of the cumulus mass more closely mimics behavior seen *in vivo* potentially indicating more normal intercellular signaling and metabolism due to L-carnitine supplementation.

Reactive oxygen species levels did not differ between treatments 4.3

In addition to its critical role in lipid metabolism, L-carnitine has been demonstrated to have potent antioxidant effects in the *in vitro* environment [97, 98]. Prior work in other domestic species has corroborated the antioxidant effect when used in gamete and embryo culture. Porcine oocytes showed a reduction in ROS levels with Wu et al. reporting a concomitant increase in levels of glutathione [107, 108]. Additionally, Catandi et al. reported that L-carnitine supplementation reduced both mitochondrial and cytosolic ROS in day 3 bovine embryos [106]. A similar effect was not observed within our study where no difference in ROS levels was

reported across all treatment groups. While no apparent change was observed in ROS levels, it is possible that the antioxidant effect of L-carnitine was masked by the addition of pyruvate to the maturation media. Pyruvate is added to maturation media to both support oocyte metabolism while also providing antioxidant effects [91, 92]. While prior reports have reported reduction in ROS in the presence of both L-carnitine and pyruvate, it may be worth investigating the impact of L-carnitine in maturation media alone. Pyruvate generally acts as a critical energy source during maturation, being passed from cumulus cells to the oocyte for oxidative phosphorylation; however, equine oocytes appear not to use supplied pyruvate as a fuel for oxidative phosphorylation during IVM [10]. Considering this, investigating L-carnitine without pyruvate in the media should have limited impact on equine COC metabolism allowing for a clearer illustration of its antioxidant effects. Regardless, while the current data does not show a significant decrease in ROS levels neither does it show an increase, indicating no detriment to L-carnitine supplementation during equine IVM.

High concentrations of L-carnitine may alter mitochondrial metabolism 4.4

L-carnitine has been demonstrated as a mitochondrial modulator capable of increasing FAO within culture [105, 107]. Based on these reports, we expected increased mitochondrial membrane potential; however, mitochondrial membrane potential was not enhanced by L-carnitine supplementation, with the 4mM treatment resulting in a significant decrease relative to the standard media treatment. The reduction in mitochondrial membrane potential suggests potential reduction in oocyte developmental competence. In other domestic species concentrations exceeding 4mM L-carnitine have been demonstrated to have detrimental impacts on oocyte quality, in particular reducing nuclear maturation and blastocyst development [103,

107-108]. While supplementation with 4mM L-carnitine did not reduce nuclear maturation and blastocyst rates were not evaluated, our data suggests that 4mM L-carnitine may have suboptimal effects on equine oocytes *in vitro*. Additionally, L-carnitine has been shown to support redistribution of mitochondria, signaling enhanced cytoplasmic maturation [107]. In equine oocytes, a heterogenous mitochondrial distribution pattern is indicative of greater cytoplasmic maturity and increased mitochondrial activity [37-39]. Quantitative analysis of mitochondrial distribution patterns found no significant difference between treatment groups. Because mitochondrial distribution is also correlated with mitochondrial function, it is unsurprising that there is no difference in distribution patterns between treatments; however, based on the reduction in mitochondrial membrane potential in the 4mM treatment, we would have expected more homogenous distribution of mitochondria. In short, despite strong evidence in other domestic species showing enhanced mitochondrial function in response to L-carnitine supplementation, equine oocyte mitochondria do not seem to respond positively to L-carnitine during culture.

L-carnitine altered consumption and production of metabolites in spent media 4.5

While there was no significant alteration in glucose, pyruvate, or lactate between treatments, there were significant changes in metabolite content when comparing media only and spent media droplets within treatments. In particular, both 2 mM and 4 mM L-carnitine treatments showed a significant increase in spent media lactate, while the untreated group showed no difference. Lactate is produced through the reduction of pyruvate by lactate dehydrogenase before being exported from the cell. When pyruvate is converted to lactate, it is not available to enter the citric acid cycle, limiting its contribution to mitochondrial ATP

production [111]. As reported by Lewis et al., equine COC demonstrate substantial lactate production, with most glucose consumption accounted for by lactate production rather than pyruvate [10]. Our observations align with this report, showing significant lactate production in the absence of significant change in pyruvate concentration in spent media. Furthermore, a strong correlation was observed between glucose consumption and lactate production supporting that glucose use in the equine COC is primarily associated with lactate production *in vitro*. Without strong evidence of pyruvate production across all treatments coupled with a strong correlation between glucose consumption and lactate production, it is likely that the equine COC is relying on other endogenous fuels to support mitochondrial metabolism during the maturation period. The significant increase in lactate production observed in L-carnitine treated groups may suggest a shift away from glycolytic production of pyruvate for the citric acid cycle, further supporting the theory that equine oocytes rely on non-glucose fuel sources during oocyte maturation; however, increased lactate production does not directly imply that FAO is elevated in response to L-carnitine supplementation. While significant lactate production was evident when comparing media only and spent media droplets, there was no significant difference in lactate production between treatment groups. This may reflect limited statistical power due to the relatively small sample size, making detection of subtle metabolic effects of L-carnitine supplementation difficult. The 4mM L-carnitine treatment also demonstrated a significant increase in glucose consumption between media only and spent media droplets, which may indicate increased glucose utilization in response to L-carnitine. This observation is consistent with the increased lactate production observed in L-carnitine supplemented groups. Although our data largely mirrors previous findings on equine COC metabolism, prior studies have demonstrated that normalizing metabolic data to DNA content can improve comparisons

between COC by accounting for variation in cumulus cell mass [112]. Because such normalization was not performed in the present study, observed difference in metabolite concentration may reflect variation in cumulus cell number rather than true treatment effect.

Limitations 4.6

Several limitations should be considered when interpreting the results of the study. First, the study population consisted of twenty grade mares between the age of 15-20 years. While mares in our population were primarily below the age of twenty, a significant decline in oocyte quality is evident in mares twenty years and older [113]. Considering that mares in our population are approaching this significant reproductive milestone, it is likely that age is a potential confounding factor in terms of success of IVM and subsequent measures of oocyte quality. Compounding on this, the obesity status of mares enrolled in this study was not assessed. Obesity in the mare has been demonstrated to result in mitochondrial metabolic aberrations of granulosa cells, reduced free L-carnitine, and lipid accumulation in oocytes and granulosa cells, potentially contributing to reduced oocyte quality [102]. Furthermore, high lipid exposure of oocytes in the obese follicular environment results in delayed maturation, oxidative stress, and mitochondrial function [114]. Considering this, it may have been valuable to consider obesity status in terms of maturation success and measures of oocyte quality. Inclusion of mare metabolic status may have helped determine whether L-carnitine supplementation could improve oocyte quality in obese animals. Additionally, metabolic analysis in this study was limited to a relatively small number of samples and was not normalized to DNA content, which may influence interpretation of results. Finally, mitochondrial function was not directly evaluated in this study.

Future Directions 4.7

Despite the limitations, the findings of this study provide a foundation for further investigation into the role of L-carnitine during equine IVM. The enhancement of cumulus expansion in response to L-carnitine suggests potentially enhanced developmental competence; however, without fertilization and subsequent evaluation of blastocyst development, a definitive assessment of developmental competence cannot be made. Therefore, future studies should incorporate ICSI to determine whether L-carnitine supplementation during IVM improves blastocyst development rates. Further exploration of metabolic changes in equine oocytes is necessary to better understand how L-carnitine affects equine oocyte metabolism. The present study lacked a direct measure of COC metabolic activity, such as a Seahorse assay, which would allow for simultaneous measurement of mitochondrial respiration and glycolytic activity. Such data could provide additional insight into the increased lactate production observed in L-carnitine treated groups. Additionally, considering the reduced mitochondrial membrane potential observed in the 4 mM treatment, it is important to determine how L-carnitine supplementation alters mitochondrial respiration. Seahorse-based metabolic analysis could also provide insight into FAO in live cells, helping determine whether L-carnitine supplementation alters lipid utilization in equine oocytes. The ideal L-carnitine dosage also requires significant consideration in future studies. While both the 2 mM and 4 mM treatment showed significant improvements in cumulus expansion, the 4 mM treatment demonstrated a reduction in mitochondrial membrane potential. Thus, future studies should further evaluate L-carnitine dosage to determine the optimal concentration of L-carnitine for equine IVM.

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