DISSERTATION

OOCYTE METABOLISM – A POTENTIAL LINK BETWEEN MARE CONDITIONS AND IMPAIRED FERTILITY

Submitted by

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ABSTRACT

OOCYTE METABOLISM – A POTENTIAL LINK BETWEEN MARE CONDITIONS AND IMPAIRED FERTILITY

Maternal advanced aging and obesity are known for negatively affecting reproductive outcomes by directly impacting the oocyte and the follicular environment, where the oocyte develops and matures. Success of early embryonic development relies on appropriate ability of the oocyte to produce energy. Whether maternal conditions of the mare impact oocyte metabolic function had not been previously determined. In the studies described throughout this dissertation, novel microsensors were utilized to quantify aerobic and anaerobic metabolism of single equine oocytes. Additional and complementing end points were obtained through high-resolution respirometry of granulosa cells and metabolomic profiling of oocytes and cumulus cells. The overarching hypothesis of this dissertation is that mare conditions known to impair fertility, namely advanced age and obesity, affect oocyte metabolism, ultimately impairing oocyte developmental potential. It was additionally hypothesized that dietary supplementation to old or obese mares would reach and affect the ovarian follicular environment and the oocyte, improving its metabolic function and quality. To test these hypotheses, a series of three projects were conducted to: 1) Investigate effects of mare advanced aging on oocyte metabolism; 2) Determine the potential of diet supplementation to old mares to improve oocyte metabolism; 3) Investigate effects of mare obesity on oocyte metabolism and the potential of diet supplementation on normalizing metabolic alterations.

Findings from these projects revealed that mare advanced aging impairs oocyte aerobic and anaerobic metabolic function, contributing to limited embryonic metabolism and development after intracytoplasmic sperm injection (ICSI). Short-term dietary supplementation to old mares with feed additives, specifically formulated to improve mitochondrial metabolism and overall equine health, was able to improve mitochondrial metabolism of granulosa cells and oocytes, promoting greater embryonic rates after ICSI in comparison to a control grain supplementation. Additionally, the findings here reported demonstrate that mare obesity promotes several alterations in the ovarian follicle, including excess of reactive oxygen species production by granulosa cells, lipid accumulation in cumulus cells and oocytes, and excessive oocyte aerobic and anaerobic metabolism. Dietary supplementation to obese mares with similar feed components mitigated many of the obesity-associated follicular changes, likely contributing to oocyte quality.

Collectively, these novel discoveries contribute to knowledge and understanding of the direct effects of maternal conditions of the mare on the ovarian follicle and oocyte, elucidating cellular mechanisms by which advanced aging and obesity disturb fertility. Furthermore, these findings reveal the benefits of dietary interventions in improving oocyte metabolism and quality. Dietary supplementation represents a non-invasive and feasible approach to tackle female subfertility. Assuredly the results presented throughout this dissertation will contribute to the equine reproduction industry, with potential to have a translational impact on the human fertility industry, by not only elucidating direct effects of maternal conditions on oocyte metabolism, but also by providing a practical method for rescuing it *in vivo*.

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DEDICATION

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CHAPTER I: HYPOTHESES AND EXPERIMENTAL AIMS

Overarching Hypothesis

Maternal conditions of females, such as advanced aging and obesity, are known for negatively affecting reproductive outcomes. This has been observed in mares and other mammalian species, including rodents, bovine and human. Several different aspects of female reproduction can be impacted by maternal conditions, but oocyte quality seems to be the most impactful. The oocyte develops and matures in the ovarian follicular environment, nurtured and supported by somatic follicular cells, namely granulosa and cumulus cells, which directly transfer nutrients to the oocyte. During development and maturation within the follicle, the oocyte goes through changes to acquire the ability to develop into an embryo after fertilization, including accumulation of energy substrates, cell machinery, protein and mRNA to be utilized during the initial cleavage divisions. Appropriate ability of the oocyte to produce energy is crucial to ensure successful early embryonic development. Technical limitations for measuring metabolic function from single oocytes have restricted the ability of research investigation over the specific effects of maternal conditions on oocyte metabolism.

In the studies described herein, a novel microsensor technology was utilized to quantify aerobic and anaerobic metabolism from single equine oocytes. Additional and complementing end points were obtained through high-resolution respirometry of granulosa cells and metabolomic profiling of oocytes and cumulus cells. Through these methods, the studies in this dissertation tested the overarching hypothesis that mare conditions known to impair fertility, namely advanced age and obesity, affect oocyte metabolism, ultimately impairing oocyte quality and its ability to successfully develop into an embryo. Furthermore, it was hypothesized that

dietary supplementation to old or obese mares with feed additives, specifically formulated to improve mitochondrial metabolism and overall equine health, would reach and affect the ovarian follicular environment and the oocyte, improving its metabolic function and quality. To test these hypotheses, I completed three research projects with experimental aims that address different aspects of the effects of mare advanced aging, obesity, and dietary supplementation on the ovarian follicular environment, oocyte metabolism and quality.

Projects

1. Investigate effects of mare advanced aging on oocyte metabolism

Advanced age is known for negatively affecting fertility in mares. A decrease in pregnancy rates after natural mating can be noticed in mares as they reach their mid teen years, with a steeper decline around twenties. Although pathologies or alterations in different segments of the reproductive tract contribute to the age-associated reduced fertility, oocytes from old mares surgically transferred into the oviducts of inseminated recipient mares still produce fewer pregnancies when compared to similar transfer of oocytes obtained from young mares. This suggests a direct effect of aging on the oocyte. Different from the male gamete that continues to go through mitosis during pubertal and adult life, oocytes are not able to regenerate their population after birth. When mammalian females are born, all the oocytes they will produce throughout their life are already present in their ovaries as primary oocytes, arrested in prophase I of meiosis I. As mammalian females go through reproductive cycles, primordial follicles are recruited to differentiate into primary, secondary and tertiary follicles, which go through growth, deviation and dominance or atresia. As these females age, the primary oocytes in their ovaries also age, becoming more prone to developmental mistakes after fertilization. Maternal aging is

known for affecting mitochondrial function of different cell types, but evidence of these effects on the oocyte are lacking in many species, especially the mare. Hence, in this project, I sought to investigate the direct effects of mare advanced aging on different aspects of oocyte metabolism and quality by testing the hypothesis below through the following experimental aims.

Hypothesis: Mare aging impairs oocyte developmental potential by altering metabolic function.

Specific Aim 1: Determine effects of mare advanced aging on abundance of main energy substrates in oocytes and cumulus cells.

Specific Aim 2: Determine effects of mare advanced aging on oocyte metabolic function and mitochondrial numbers.

Specific Aim 3: Determine effects of mare advanced aging on *in vitro*-produced embryo metabolic function and development.

In this project, cumulus-oocyte complex samples were obtained from preovulatory follicles of young or old mares and cultured *in vitro* until completion of meiosis I. Mature oocytes were either assayed for metabolite composition, along with their respective cumulus cells, by liquid and gas chromatography mass spectrometry, for metabolic function using oxygen and pH microsensors, or fertilized through intracytoplasmic sperm injection (ICSI) for embryo production. The results obtained in this study demonstrate major impacts of aging on equine oocyte metabolic function and capacity, early-embryo aerobic metabolism and embryonic production after ICSI. These findings not only support the proposed hypothesis but elucidate some of the cellular mechanisms that link maternal aging and reduced oocyte quality, allowing for development of interventions aiming to correct oocyte metabolic dysfunction, as investigated in my second project.

2. Determine the potential of diet supplementation to old mares in improving oocyte metabolism

Impaired mitochondrial function is one of the hallmarks of age-associated cellular metabolic dysfunction due to reduced expression of antioxidant enzymes, increased reactive oxygen species (ROS) production, oxidative damage and stress. Results from Project 1 demonstrated that mitochondrial dysfunction also happens in oocytes from old mares, contributing to their limited ability to develop into embryos after fertilization. A possible approach to ameliorate cellular metabolism *in vivo* is through dietary supplementation with nutraceuticals targeting mitochondria, including antioxidants to combat ROS generation and oxidative damage, co-enzymes and co-factors that participate in mitochondrial metabolism, and energy supplies to be utilized for ATP production. Thus far, no specific diet supplementation guidelines are available for aged women, let alone mares undergoing fertility issues. After clinical observations in our commercial assisted reproduction program of improved pregnancy rates from transfer of oocytes obtained from old mares supplemented with commercially available feed additives (Platinum Performance, Inc.), we decided to assess the direct effects of similar supplements on the ovarian follicular environment and oocyte metabolism through a series of controlled studies. In this project, a combination of compounds designed to promote overall wellness and cellular health was utilized with the main objective of supporting oocyte quality (Reproductive Support Supplements). During consecutive studies, the supplement formulation was altered slightly in an attempt to identify the most beneficial ingredients. Herein, I investigated the potential of dietary supplementation fed to old mares on directly reaching and altering the follicular environment, contributing to oocyte metabolism and quality by testing the hypothesis below through the following experimental aims.

Hypothesis: Dietary supplements fed to old mares for a limited time improve oocyte metabolic function and developmental potential.

Specific Aim 1: Determine effects of supplementation with Reproductive Support Supplements (RSS) to old mares on granulosa cell, oocyte, and early embryo metabolism, compared to a control grain mix supplementation.

Specific Aim 2: Determine effects of supplementation with RSS to old mares on lipid abundance in circulation, in the ovarian follicle and in the oocyte.

Specific Aim 3: Determine effect of supplementation with RSS to old mares on oocyte developmental potential after ICSI.

In this project, samples were obtained from preovulatory follicles of old mares supplemented with different feed additives during consecutive breeding seasons. For the first experiment, a group of old mares was supplemented for approximately eight weeks with RSS, while a similar group of mares was fed a control grain-based supplementation for the same period. In the second experiment, samples were collected from old mares before and after approximately eight weeks of supplementation with a slightly modified RSS formulation containing fewer antioxidants. Finally, in the third experiment, three groups of old mares were used, one fed a control grain-based supplementation, one fed the same modified RSS formulation from the second experiment, and one fed RSS modified to contain greater proportion of omega-6 than omega-3 polyunsaturated fatty acids. Through these consecutive experiments we were able to observe direct effects of short-term RSS supplementation in the old mare ovarian follicle and oocyte, including improved granulosa cell and oocyte mitochondrial metabolism, reduced lipid abundance in oocytes and follicular fluid, and greater oocyte developmental potential compared to control supplementation or pre-supplementation samples. These results support the proposed hypothesis and provide a feasible approach for improving reproductive outcomes of old broodmares facing fertility limitations. Our exciting and novel findings led us to explore, in my

third project, the ability of similar feed supplements to correct follicular cell metabolic disturbances promoted by mare obesity. Although being a distinct condition from aging, maternal obesity is known for also leading to metabolic disturbances of the oocyte in other species, and to lipid accumulation in the follicle and oocyte in mares.

3. *Investigate effects of mare obesity on oocyte metabolism and the potential of diet supplementation on normalizing metabolic alterations*

Obesity is a prevalent condition in both human and equine populations and can lead to systemic metabolic and reproductive disturbances in women and mares. With obesity, elevated circulating lipids also accumulate in the ovarian follicular environment and oocyte, possibly leading to mitochondrial dysfunction and oxidative stress. Feed additives, including L-carnitine, antioxidants and chromium, seem to contribute to correcting obesity-induced cellular metabolic disturbances systemically, but potential effects on metabolic function of ovarian follicular cells have not been assessed. After determining in Project 2 that dietary supplements fed to old mares can reach and improve metabolism of the oocyte, I sought to examine the capability of a similar intervention in mitigating oocyte and follicular cell metabolic alterations promoted by mare obesity. To test the hypothesis below, I conducted the following experimental aims.

Hypothesis: Mare obesity promotes lipid accumulation and altered metabolic function of oocytes and follicular cells, which can be corrected through short-term dietary supplementation.

Specific Aim 1: Determine effects of mare obesity on granulosa cell mitochondrial function, and the potential of dietary supplementation to obese mares in normalizing alterations.

Specific Aim 2: Determine effects of mare obesity on lipid abundance in the ovarian follicle, cumulus cells and oocyte, and the potential of dietary supplementation to obese mares in normalizing alterations.

Specific Aim 3: Determine effects of mare obesity on oocyte metabolic function and the potential of dietary supplementation to obese mares in normalizing alterations.

Samples were collected from preovulatory follicles of normal-weight, obese and obese mares supplemented with feed additives similar to RSS but modified to target metabolic changes induced by obesity. Results from this study evidence substantial impacts of mare obesity on metabolic function and lipid accumulation of follicular cells and oocytes. Most of these impacts were normalized or mitigated through dietary supplements fed to obese mares, demonstrating once again the strength of this non-invasive intervention in modifying the ovarian follicle and oocyte. Our findings support the proposed hypothesis, unravel specific obesity-induced metabolic effects on the ovarian follicle and oocyte, and suggest the benefits of specifically formulated feed additives on the ovarian follicular environment of obese mares.

Outcomes from these three projects, described in more detail in the following chapters, contribute to knowledge and understanding of the direct effects of mare conditions known for disturbing fertility on the ovarian follicle and oocyte. Moreover, the findings herein presented elucidate the beneficial effects of dietary interventions in improving oocyte quality and reproductive success. Diet supplementation is a non-invasive and feasible approach for horse owners to attempt with subfertile mares. Assuredly these results will contribute to the equine reproduction industry, and potentially have translational contributions to the human fertility industry.

CHAPTER II: EQUINE MATERNAL AGING AFFECTS OOCYTE LIPID CONTENT, METABOLIC FUNCTION AND DEVELOPMENTAL POTENTIAL[1]

Summary

Advanced maternal age is associated with a decline in fertility and oocyte quality. We used novel metabolic microsensors to assess effects of mare age on single oocyte and embryo metabolic function, which has not yet been similarly investigated in mammalian species. We hypothesized that equine maternal aging affects the metabolic function of oocytes and in vitro-produced early embryos, oocyte mitochondrial DNA (mtDNA) copy number, and relative abundance of metabolites involved in energy metabolism in oocytes and cumulus cells. Samples were collected from preovulatory follicles from young (≤14 years) and old (≥20 years) mares. Relative abundance of metabolites in metaphase II oocytes (MII) and their respective cumulus cells, detected by liquid and gas chromatography coupled to mass spectrometry, revealed that free fatty acids were less abundant in oocytes and more abundant in cumulus cells from old versus young mares. Quantification of aerobic and anaerobic metabolism, respectively measured as oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in a microchamber containing oxygen and pH microsensors, demonstrated reduced metabolic function and capacity in oocytes and day-2 embryos originating from oocytes of old when compared to young mares. In mature oocytes, mtDNA was quantified by real-time PCR, was not different between the age groups and not indicative of mitochondrial function. Significantly

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more sperm-injected oocytes from young than old mares resulted in blastocysts. Our results demonstrate a decline in oocyte and embryo metabolic activity that potentially contributes to the impaired developmental competence and fertility in aged females.

Keywords: Age, Mare, Oocyte, Embryo, Metabolism

Introduction

Equine maternal aging is associated with reduced fertility, lower pregnancy rates, and more early pregnancy losses (Carnevale & Ginther, 1992; Carnevale *et al.*, 2005; Morel *et al.*, 2005; Allen *et al.*, 2007). Multiple factors contribute to the impaired fertility associated with aging, such as uterine dysfunction (Carnevale & Ginther, 1992), reduced ovarian follicle numbers (Carnevale *et al.*, 1993; Cuervo-Arango *et al.*, 2019), and a decrease in oocyte quality (Carnevale & Ginther, 1995; Hendriks *et al.*, 2015). In mares, maternal aging contributes to an increase in the incidence of altered oocyte morphology (Altermatt *et al.*, 2009), spindle abnormalities and chromosome misalignment (Rizzo *et al.*, 2018), as well as to a decline in mitochondrial DNA (mtDNA) copy numbers during maturation (Rambags *et al.*, 2014; Campos-Chillon *et al.*, 2015).

Mitochondria are the main providers of energy for cellular processes during oocyte maturation and early embryonic development through oxidative phosphorylation of adenosine diphosphate, utilizing substrates provided by cumulus cells (May-Panloup *et al.*, 2007; Gardner & Wale, 2013). Oocyte mitochondrial content, estimated by mtDNA copy number, greatly increases during maturation (Hendriks *et al.*, 2015; Lamas-Toranzo *et al.*, 2018); then, mitochondrial replication arrests at early cleavage stages and restarts at blastocyst formation in

equine embryos (Hendriks *et al.*, 2019). Both mitochondrial content and activity in the oocyte are critical for successful early embryo development and can be indicators of developmental potential (Bentov *et al.*, 2011). Alterations in mtDNA copy numbers have been related to developmental success, maternal age, and in vitro culture methods (Rambags *et al.*, 2006; Kameyama *et al.*, 2007; Diez-Juan *et al.*, 2015; Hendriks *et al.*, 2015; Pasquariello *et al.*, 2019), although conflicting results and controversy still surround the topic (Viotti *et al.*, 2017; Cecchino & Garcia-Velasco, 2019).

Mitochondrial function in oocytes and embryos has been described through a variety of methods, most of which are either invasive or require expensive equipment or a long time frame, making them unsuitable for clinical application (Sugimura *et al.*, 2012; Rambags *et al.*, 2014; Hashimoto *et al.*, 2017; Pasquariello *et al.*, 2019). Such methods are also limited in terms of data interpretation, as they do not provide actual quantification of mitochondrial activity. When studying intact cells, the best measure of mitochondrial function is the assessment of cellular respiration by quantification of oxygen consumption rate (OCR), qualified by the addition of distinct mitochondria inhibitors and uncouplers (Brand & Nicholls, 2011).

Clark-type oxygen sensors are electrochemical-based sensors capable of measuring changes in dissolved oxygen concentration from media containing biological samples and, therefore, can be used for monitoring aerobic metabolism of single mammalian embryos and oocytes (Lopes *et al.*, 2007; Tejera *et al.*, 2011; Obeidat *et al.*, 2018). Anaerobic glycolysis is routinely estimated through measurement of extracellular acidification rate (ECAR) of the surrounding media (TeSlaa & Teitell, 2014), as a proton co-exported from cells with lactate generated from anaerobic glycolysis is the main contributor to media acidification, despite some participation of other metabolic processes such as carbon dioxide production by the tricarboxylic

acid (TCA) cycle (Mookerjee *et al.*, 2015). Thus, the addition of a pH sensor in a microchamber containing the oxygen sensor allows estimation of both aerobic and anaerobic metabolism (Obeidat *et al.*, 2019a).

Studies with human oocytes and embryos have reported positive correlations between OCR, embryonic development, and fertility outcomes (Tejera *et al.*, 2011; Yamanaka *et al.*, 2011; Hashimoto *et al.*, 2017). However, effects of maternal aging on individual oocyte and embryo OCR and ECAR have not been assessed in any mammalian species. Additionally, the relationship between mitochondrial function measurements and mtDNA copy number has not been investigated in individual oocytes. The mare is a potential animal model to study maternal aging in women (Carnevale, 2008). We hypothesized that equine maternal aging impairs oocyte and early embryo metabolic parameters and function. Using microsensors, we assessed the metabolic function of individual metaphase II (MII) oocytes and intracytoplasmic sperm injection (ICSI)-produced early embryos. Functional endpoints were compared to oocyte mtDNA content. Oocytes and cumulus cells from similar age groups of mares were used to compare the relative abundance of metabolites, involved in energy metabolism and storage. Finally, oocytes were injected with sperm to determine the competence of oocytes from young and old mares to develop into blastocysts.

Materials and Methods

Animals and experimental design

Colorado State University's Institutional Animal Care and Use Committee approved all the procedures performed in this study. Samples were collected from similar groups of nonlactating mares of light-horse breeds, with some of the same mares used during three

breeding seasons. For oocyte and cumulus cell metabolite quantification, samples were collected between June and August of 2016, from young mares [Young, 7-11 years (mean 10), n=8] and old mares [Old, 20-26 (mean 22.4), n=10]. For oocyte and early embryo metabolic assays, young mares [Young, 6-13 years (mean 9.3), n=7] and old mares [Old, 20-27 years (mean 23.9), n=8] were used for sample collections between June and August of 2018. Oocytes were collected from mares in 2018 and 2019 [Young, 6-14 years (mean 10.1), n=13; Old, 20-28 years (mean 24.4), n=11] to compare cleavage and blastocyst rates after ICSI; samples were collected between June and August. Mares were housed in dry lot paddocks with access to covered shelters and fed a mixture of grass and alfalfa hay; mineral salt and water were provided ad libitum. The experimental design was a prospective observational cohort study.

Oocyte collection and maturation

Mares' reproductive tracts were examined by transrectal ultrasonography with 7.5 MHz linear probe to assess ovarian activity. Follicular maturation was induced when a dominant follicle approximately 35 mm in diameter and endometrial edema indicative of estrus were observed. Human chorionic gonadotropin (2000 IU, intravenous; Chorulon, Merck Animal Health, Madison, NJ) and deslorelin acetate in an aqueous base (0.75 mg, intramuscular; Precision Pharmacy, Bakersfield, CA) were administered at 23±2 h (for metabolomics analyses) or 16±2 h (for metabolism assays and embryo production) before oocytes were collected by transvaginal, ultrasound-guided follicular aspiration of the dominant follicles or follicles, as described (Carnevale, 2016). Only Recovered cumulus oocyte complexes (COCs) were incubated in tissue culture media 199 with Earle’s salts (GibcoTM, Thermo Fisher, Waltham, MA) with additions of 10% fetal calf serum, 25 μg/ml of gentamicin, and 0.2 mM pyruvate at 38.2°C in an atmosphere of 5% CO_2 and air for 19±2 h (metabolomic analyses of COCs) or 26±2

h (oocyte metabolic assays and ICSI). Oocyte maturation was considered complete at the time of oocyte use, approximately 42 h after the administration of induction drugs to mares, which is the timeline used for fertilization of equine oocytes from dominant follicles (Carnevale, 2016). After culture, oocytes were denuded of cumulus cells by sequential pipetting in a MOPS-buffered medium (G-MOPS™, Vitrolife, Englewood, CO) with 0.04% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO) and hyaluronidase (200 IU/mL; Sigma-Aldrich) and evaluated to confirm complete removal of cumulus cells. Extrusion of the first polar body was confirmed. For electrochemical measurements of basal and maximal oxygen consumption rates (OCR) and extracellular acidification rates (ECAR), oocytes were placed in a MOPS-buffered medium (G-MOPS) with 0.04% BSA at 38.2°C until metabolism assays. For metabolomics analyses, individual oocytes and their respective cumulus cells were fixed separately in 50% methanol solution and stored in glass vials at -80ºC until analyses.

Oocyte and cumulus cell metabolite extraction and detection using liquid and gas chromatography coupled to mass spectrometry

For the experiment, one set of samples (oocyte and corresponding cumulus cells) was collected per mare. Oocytes or cumulus cells, frozen individually at -80ºC, were thawed to 4ºC before the addition of 250 µL of 100% methanol. Samples were then sonicated in a QSonica ultrasonic processor at 65% amplitude for 10 min, before being vortexed for 2 h at 4ºC. After centrifugation at 3000 x g at 4ºC, two individual aliquots of 120 µL of extract were transferred into 2-mL glass vials and dried under nitrogen gas for mass spectrometry analyses by liquid chromatography (LC-MS) and gas chromatography (GC-MS). The number of cumulus cells per sample was not known, so the remaining pellet was used to estimate biomass through

quantification of protein by reconstitution with urea and measurement of absorbance at 280 nm using a NanoDrop™ spectrophotometer (Thermo Fisher).

For LC-MS analysis, the cumulus cell extract was resuspended in volumes proportional to the protein content (5 μL of 100% methanol was used per 5 μg/μL of protein content) with a minimum volume of 25 μL. Oocyte samples had a protein concentration less than 5 μg/μL and were resuspended in 25 μL of 100% methanol. Two μL of the suspensions were injected onto a ACQUITY UPLC system (Waters, Milford, MA) in randomized order and separated using a ACUITY UPLC CSH Phenyl Hexyl column (1.7 μM, 1.0 x 100 mm) (Waters), using a gradient from solvent A (A) (2 mM ammonium hydroxide, 0.1% formic acid) to solvent B (B) (99.9% acetonitrile, 0.1% formic acid). Injections were made in 100% A, held at 100% A for 1 min, ramped to 98% B over 12 min, held at 98% B for 3 min, and then returned to start conditions over 0.05 min and allowed to re-equilibrate for 3.95 min, with a 200 μL/min constant flow rate. The column and samples were held at 65ºC and 6ºC, respectively. The column eluent was infused into a Xevo G2 Q-TOF-MS (Waters) with an electrospray source in positive mode, scanning 50-2000 m/z at 0.2 s per scan, alternating between MS (6V collision energy) and MS^E mode (15-30V ramp). Calibration was performed using sodium iodide with 1 ppm mass accuracy. The capillary voltage was held at 2200 V, source temperature at 150ºC, and nitrogen desolvation temperature at 350ºC with a flow rate of 800 L/h.

For GC-MS analysis, the extract was resuspended in pyridine containing 25 mg/mL of methoxyamine hydrochloride (5 μL per 5 μg/μL of protein content for cumulus cells and 25 μL for oocytes), incubated at 60ºC for 60 min, vigorously vortexed for 30 s, sonicated for 10 min, and incubated for an additional 60 min at 60ºC. Additions of the same volume of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS, Thermo

Fisher) were made, and samples were vigorously vortexed for 30 s, then incubated at 60ºC for 30 min. Metabolites were detected using a TRACE 1310 GC coupled to a ISQ™ mass spectrometer (ThermoFisher). One μL of the samples were injected at 10:1 split ratio to a 30 m TG-5MS column (Thermo Fisher, 0.25 mm i.d., 0.25 μm film thickness) with a 1.2 mL/min helium gas flow rate. The GC inlet was held at 285ºC. The oven program started at 80ºC for 30 s, followed by a ramp of 15ºC/min to 330ºC, and an 8 min hold. Masses between 50-650 m/z were scanned at 5 scans/s under electron impact ionization. Transfer line and ion source were held at 300 ºC and 260ºC, respectively.

For each sample, raw data files were converted to a computable document format (CDF), and matrix of molecular features, as defined by retention time and mass (m/z), was generated using XCMS in R software (BMC Bioinformatics) for feature detection and alignment (Smith *et al.*, 2006). To account for the variance in cell numbers and protein content from cumulus cell samples, raw peak areas were first normalized to total protein in the sample. Oocyte samples were not normalized to protein content, as a constant volume was used for resuspension, and each sample contained a single cell. Peak areas were also subsequently quantile normalized in R. Outlier injections were detected based on total signal and PC1 of principle component analysis. Features were grouped using RAMClustR (Broeckling *et al.*, 2014), which groups features into spectra based coelution and covariance across the full dataset, whereby spectra are used to determine the identity of observed compounds in the experiment. The peak areas for each feature in a spectrum were condensed via the weighted mean based on spectral matching to in-house, NISTv14, Golm, HMDB and LipidMaps 1-SToP libraries (Broeckling *et al.*, 2016), and Metlin metabolite databases. For this study, we focused on interpretation of glucose, pyruvate, lactate and free fatty acids from the GC-MS data, as the primary energy substrates for oocyte maturation

and early embryonic development. For LC-MS data, triglycerides (TG) composition was the focus of this study, as these are the main components of cellular energy storage.

In vitro embryo production

Prior to ICSI, oocytes were collected, matured and denuded of cumulus cells as described above. Frozen-thawed sperm from a single ejaculate from one stallion were used for ICSI. Approximately one-tenth of a 0.25-mL straw of frozen semen was cut under liquid nitrogen and thawed by placing directly in 1mL of MOPS-buffered medium (G-MOPS) with 0.04% BSA at 38.2ºC. Prior to injection, one sperm was selected and prepared for sperm injection as previously described (Altermatt *et al.*, 2009). ICSI was performed in MOPS-buffered medium (G-MOPS) with 0.04% BSA using a micromanipulator (Narishige Group, Amityville, NY) and a piezo-driven injection system (Prime Tech, Japan), after which presumptive zygotes were placed into embryo culture medium (global®, LifeGlobal Group, Guilford, CT) in individual 30-µL droplets under paraffin oil (OVOIL™, Vitrolife) and incubated at 38.2ºC in 5% O_2, 7% CO_2 and 88% N_2. At 2 days (44-56 h, mean of 51.5 h after ICSI), early embryos with normal morphology and 2 to 8 cells (mean of 4 cells) were used for the measurement of basal OCR and ECAR. Embryos followed for blastocyst development were moved at 5 days after ICSI into individual 30-µl droplets of a second culture medium (global® for Fertilization, LifeGlobal Group). Embryos were observed daily until formation of a blastocyst or degeneration.

Oocyte and early embryo OCR and ECAR assays

Metabolic analyses of oocytes and early embryos were performed using a microchamber with electrochemical-based oxygen and pH sensors previously described in detail (Obeidat *et al.*, 2019a, b). One or two samples per mare were analyzed. Briefly, for each oocyte or early embryo assay, the microchamber was filled with 120 µL of MOPS-buffered medium (G-MOPS) with

0.04% BSA, overlaid with 120 μL of paraffin oil (OVOIL) to limit the chamber from atmospheric oxygen diffusion. The three-electrode oxygen sensor was connected to a potentiostat (Quadstat EA 164H, eDAQ Inc., Colorado Springs, CO) that applied voltage to the sensor and monitored the decrease in oxygen reduction current over time. The applied potential during all amperometric oxygen assays was set at -0.6V. The pH sensor was connected to a custom-made Ina333 instrumentation amplifier circuit that measured the change in voltage. The starting (room air saturated) oxygen concentration and pH of the medium was measured as baseline current for 2 min and calibrated as previously described in detail (Obeidat *et al.*, 2019a). The rate of change in chamber oxygen and pH from baseline values over time (following addition of sample) were used for calculations of sample OCR and ECAR, respectively.

After measuring oxygen and pH baselines, individual oocytes or early embryos were transferred into the microchamber and placed on top of the oxygen sensor. Basal OCR was assayed for 5 min, followed by ECAR assessment for 2 min. To determine respiration related to proton leak and stimulate maximal ECAR, a subset of 10 oocytes (n=3 from Young, n=7 from Old) was exposed to 1 μM oligomycin, an ATP-synthase inhibitor; OCR was assessed for 5 min and ECAR for 2 min. Because oxygen consumption related to proton leak was so small, as previously demonstrated by our group (Obeidat *et al.*, 2018), subsequent oocyte assays did not include the addition of oligomycin.

Our laboratory has validated that the maximal respiratory capacity of bovine oocytes can be achieved by titration of protonophore uncoupler agents (Obeidat *et al.*, 2018). Three titrations of 1 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, Sigma-Aldrich) were performed during the equine oocyte assays, each followed by OCR measurement for 5 min and ECAR for 2 min. Maximal OCR was defined as the highest stable value observed during CCCP titrations.

After metabolic readings, individual oocytes were stored at -80°C for subsequent mitochondrial DNA (mtDNA) quantification.

Oocyte mitochondrial DNA content absolute quantification

Mitochondrial DNA (mtDNA) content of single oocytes (one or two samples per mare) was quantified by real-time PCR (qPCR) as previously described (Pasquariello *et al.*, 2019) using kits and supplies from one source (Qiagen, German-town, MD) unless otherwise noted. Briefly, DNA extraction of individually cryopreserved oocytes was performed using the QIAamp DNA micro kit according to the manufacturer's protocol with carrier RNA (1 μg) added to each sample. The DNA sample was eluted with 50 μL of Buffer AE (supplied with the kit) and analyzed for RT-qPCR using an absolute quantification assay. To prepare quantification standards, a 1096 bp fragment of the I-rRNA region of equine mtDNA was amplified by PCR using the LongRange PCR kit and a primer pair (5'-AGCAATTTCGGTTGGGGTGA-3' and 5'-GCTCGGTTGGTTTCGGCTAA-3') designed using Primer-BLAST (NBCI), then purified using the Qiaquick PCR purification kit and cloned using the Qiagen PCR cloning kit. Plasmid DNA containing the amplified mtDNA fragment was purified from bacteria using the Qiaprep miniprep kit. A standard curve was generated by using seven tenfold serial dilutions (10^7 to 10 copies), and standard curve correlation coefficients were greater than 0.98. Real-time quantitative PCR using the primer pair 5'-ATGGTTTGTGCTACTGCTCG-3' and 5'-GCCCTAACCCTGGCCTTAAC-3', designed with Primer-BLAST (NCBI), was run in triplicate for each standard dilution and sample in 10-μL reactions using PowerUp SYBR Green master mix (Applied Biosystems, Foster City, CA), a LightCycler 480II (Roche Applied Science, Indianapolis, IN) and the program of amplification: 50°C for 2 min for the first cycle; 95°C for 2 min for the second cycle; 95°C for 15 s and 60°C for 1 min for 40 cycles; a melting curve was

run to assess specificity of the primers. Samples and standard curve were run on the same plate. Copy numbers of mtDNA were generated from the equation of Ct value against copy number for the corresponding standard curve.

Statistical analyses

Statistical analyses were completed using GraphPad Prism 8.0.2 (GraphPad Software, Inc., San Diego, CA). Student's *t*-tests were used to compare oocyte and early embryo continuous data, including relative abundance of metabolites, OCR and ECAR. Mann-Whitney tests were used to compare metabolomics data that failed the Shapiro-Wilk test for normality. Fisher's exact tests were used to compare cleavage and blastocyst rates. For OCR and ECAR comparisons of different developmental stages (oocytes and embryos), one-way ANOVA was used, followed by a post-hoc Tukey's HSD to determine the source of any differences. Differences were considered significant at $P < 0.05$. Relative abundances of metabolites are presented in box plots (median, first and third quartiles) with whiskers from minimum to maximum values. The remaining results are presented as mean ± SEM.

Results

Effects of maternal age on oocyte and cumulus cell metabolite composition

Metaphase II oocytes and their respective cumulus cells from Young (n=8) and Old (n=10) were evaluated by LC-MS and GC-MS for metabolite composition. One sample was obtained per mare, with cumulus cells and oocytes obtained from the same follicle. Relative abundance of glucose, pyruvate and lactate were similar between the two age groups for oocytes ($P \geq 0.6$) and cumulus cells ($P \geq 0.2$; Figure 1A-F). Relative abundance of total FFA were greater in oocytes from Young than Old, respectively ($P=0.048$; Figure 1G), but not in cumulus cells

(P=0.08; Figure 1H). Total abundance of TG in oocytes and cumulus cells were similar between Young and Old (P≥0.2; Figure 1I, 1J); the relative abundance of each TG identified via LC-MS is included in Supplementary Table 1 (Appendix I).

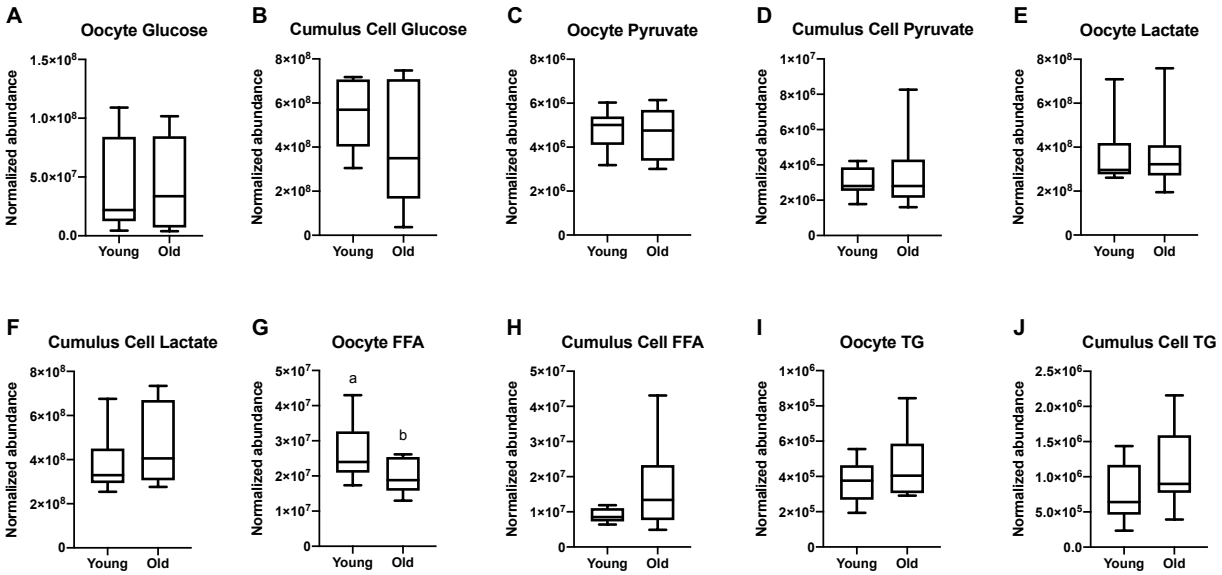

Figure 1: Relative abundance of metabolites in metaphase II oocytes and the respective cumulus cells from young and old mares. Relative abundance of glucose in (A) oocytes and (B) cumulus cells, pyruvate in (C) oocytes and (D) cumulus cells, lactate in (E) oocytes and (F) cumulus cells, total free fatty acids (FFA) in (G) oocytes and (H) cumulus cells (P=0.08), and total triglycerides (TG) in (I) oocytes and (J) cumulus cells. Single samples were analyzed from Young (n=8 mares) and Old (n=10 mares). Box plots present median, first and third quartiles, with whiskers from minimum to maximum values. Different superscripts indicate differences at $P < 0.05$ (Student's *t* test).

Four FFA were identified in oocytes and cumulus cells (Figure 2A and 2B). Although only the relative abundance of palmitic acid (C16:0) was higher (P=0.05) in oocytes, the general tendency was for all of FFA to be numerically higher in oocytes from Young than Old (stearic acid, C18:0, P=0.06; oleic acid, C18:1, P=0.1; linoleic acid, C18:2, P=0.1; Figure 2A). In contrast, the normalized abundance of linoleic acid was higher (P=0.01) in cumulus cells of Old when compared to Young, with similar relationship observed for most other FFA (palmitic acid, P=0.07; stearic acid, P=0.6; oleic acid, P=0.1; Figure 2B).

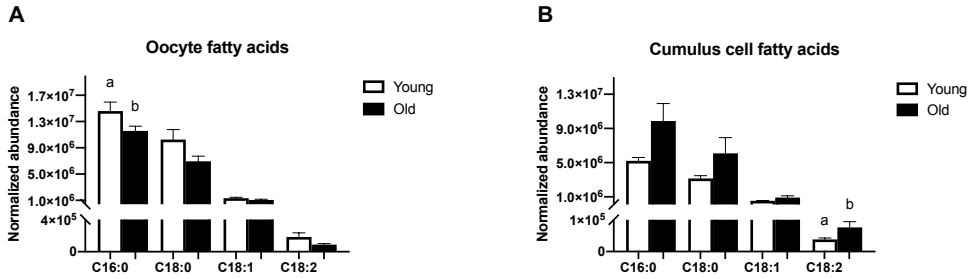

Figure 2: Relative abundance of free fatty acid species in metaphase II oocytes and the respective cumulus cells from young and old mares. Relative abundance of palmitic acid (C16:0) in (A) oocytes and (B) cumulus cells (P=0.07), stearic acid (C18:0) in (A) oocytes (P=0.06) and (B) cumulus cells, oleic acid (18:1) in (A) oocytes and (B) cumulus cells, and linoleic acid (18:2) in (A) oocytes and (B) cumulus cells. Single samples collected from Young (n=8 mares) and Old (n=10 mares). Barcharts present mean ± SEM. Different superscripts indicate differences at $P < 0.05$ (Student's *t* test and Mann-Whitney test).

Effects of maternal age on oocyte metabolic function and mtDNA copy number

Oocytes from Young (n=9) and Old (n=14) were assayed for OCR and ECAR. Basal OCR was higher for oocytes from Young (1.8 ± 0.2 fmolO_2/s/oocyte) than Old (1.4 ± 0.1 fmolO_2/s/oocyte, P=0.02; Figure 3A). Higher rates of maximal OCR were also expressed by oocytes from Young (2.7 ± 0.2 fmolO_2/s/oocyte) when compared to Old (2.1 ± 0.1 fmolO_2/s/oocyte, P=0.007; Figure 3B). Mitochondrial efficiency, calculated as basal OCR/maximal OCR, represents the proportion of maximal respiratory capacity that a cell utilizes during basal metabolism. Mitochondrial reserve capacity, calculated as maximal OCR – basal OCR, is an indicator of the cellular ability to respond to increased energy demands. Both parameters were similar between groups ($P \geq 0.4$). Proton leak OCR, achieved after addition of oligomycin, was not different among oocytes from Young and Old (0.02 ± 0.01 and 0.01 ± 0.004 fmolO_2/s/oocyte, respectively, P=0.8; Figure 3C). No difference was noted in mtDNA copy number between oocytes from Young (n=10) and Old (n=13) ($5.6 \times 10^5 \pm 0.4 \times 10^5$ and $6.2 \times 10^5 \pm 0.4 \times 10^5$, respectively, P=0.3; Figure 3D).

Basal ECAR, an estimation of anaerobic glycolysis, was higher for oocytes from Young (20.8 ± 1.7 mpH/min/oocyte) than Old (15 ± 1.6 mpH/min/oocyte, P=0.03; Figure 3E). Maximal ECAR, stimulated by addition of oligomycin, was also higher (P=0.006) for Young (27.8 ± 2.9 mpH/min/oocyte) compared to Old (18.1 ± 1.2 mpH/min/oocyte, P=0.006; Figure 3F). The basal rate of glycolysis relative to oxidative phosphorylation, expressed as the ECAR/OCR ratio, was not different between groups (P=0.7; Figure 3G), indicating no effect of maternal aging on the relative contributions of anaerobic and aerobic metabolism to oocyte energy supply.

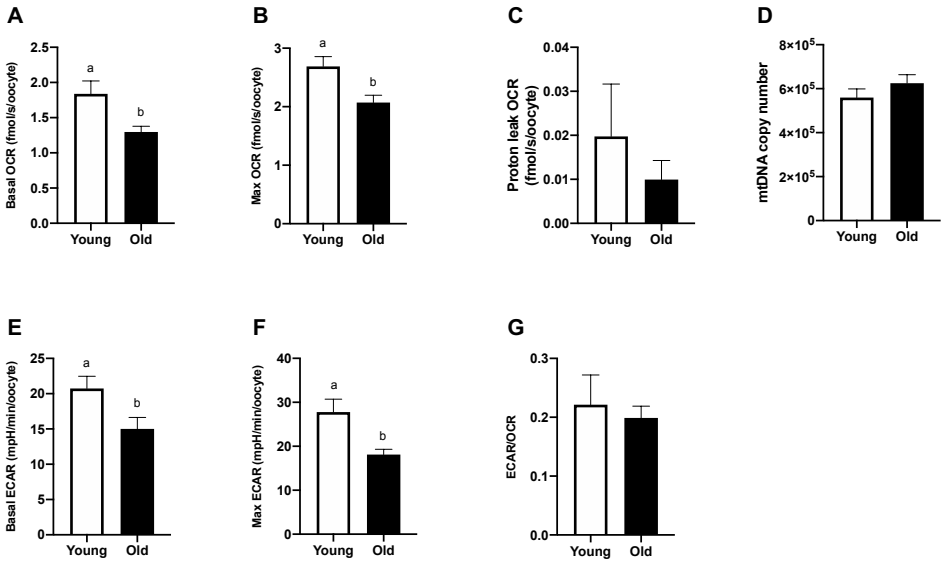

Figure 3: Metabolic function and mitochondrial DNA copy number of metaphase II oocytes from young and old mares. (A) Basal OCR (Young, n=9 from 7 mares; Old, n=14 from 8 mares), (B) maximal OCR induced by CCCP titrations (Young, n=9 from 7 mares; Old, n=14 from 8 mares), (C) proton leak OCR induced by addition of oligomycin (Young, n=3 from 3 mares; Old, n=7 from 5 mares), (D) mtDNA (Young, n=10 from 7 mares; Old, n=13 from 8 mares), (E) basal ECAR (Young, n=9 from 7 mares; Old, n=14 from 8 mares), (F) maximal ECAR induced by oligomycin titrations (Young, n=3 from 3 mares; Old, n=7 from 5 mares), and (G) proportion of basal glycolytic rate over oxidative phosphorylation rate (Young, n=9 from 7 mares; Old, n=14 from 8 mares). Barcharts present mean ± SEM. Different superscripts indicate differences at $P < 0.05$ (Student's *t* test).

Effects of maternal age on early embryo metabolic function

Basal OCR and ECAR were assessed in day-2 embryos from Young (n=8) and Old (n=10). Basal OCR was higher for embryos from Young (3.8 ± 0.1 fmolO_2/s/embryo) than Old (3.2 ± 0.2 fmolO_2/s/embryo, P=0.05; Figure 4A). Basal ECAR was similar between groups (36.9 ± 2.7, 38.5 ± 2.1 mpH/min/embryo, P=0.7; Figure 4B). The ECAR/OCR ratio was similar for embryos from Young and Old (P=0.1; Figure 4C).

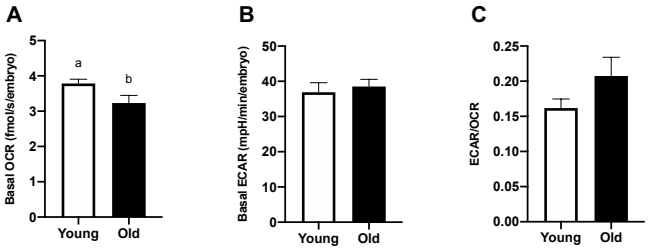

Figure 4: Metabolic function of in vitro-produced, day-2 embryos from young and old mares. (A) Basal OCR (Young, n=8 from 7 mares; Old, n=10 from 8 mares), (B) basal ECAR (Young, n=7 from 7 mares; Old, n=3 from 3 mares) and (C) proportion of glycolytic rate over oxidative phosphorylation rate (Young, n=7 from 7 mares; Old, n=3 from 3 mares). Barcharts present mean ± SEM. Different superscripts indicate differences at $P < 0.05$ (Student's *t* test).

Basal OCR in early embryos was consistently higher than basal and maximal OCR in oocytes from Young or Old (P≤0.0001; Figure 5A). Similarly, basal ECAR in embryos was higher than in oocytes from Young or Old (P≤0.0001; Figure 5B), consistent with an increase in energy demands from both anaerobic and aerobic pathways during early embryo development. Average cell numbers of the day-2 embryos were not different between Young and Old (4.4 ± 0.5, 5 ± 0.4 cells, P=0.4).

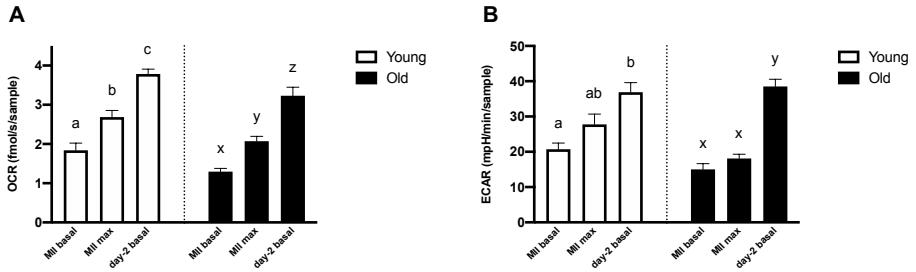

Figure 5: Metabolic function of metaphase II oocytes and in vitro-produced, day-2 embryos compared within the same age group. (A) Basal and maximal OCR of MII oocytes and basal OCR of day-2 embryos. (B) Basal and maximal ECAR of MII oocytes and basal ECAR of day-2 embryos. Barcharts present mean ± SEM. Different superscripts indicate differences within the same age group (^{abc}Young and ^{xyz}Old) at $P < 0.05$ (one-way ANOVA, post-hoc Tukey's HSD).

Effects of maternal age on embryo development after ICSI

The number of sperm-injected oocytes that cleaved into at least two cells at 1 or 2 days after ICSI was similar between groups (Young, 25/29, 86% and Old, 22/24, 92%; Figure 6); however, embryonic development to the blastocyst stage at day 7 or 8 after ICSI was greater (P=0.04) for Young than Old per sperm-injected oocyte (Young, 14/29, 48% and Old, 5/24, 21%; Figure 6) or per cleaved embryo (Young, 14/25, 56% and Old, 5/22, 23%).

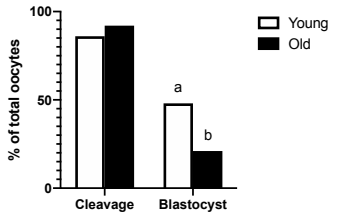

Figure 6: Percentage of sperm injected oocytes that cleaved into at least 2 cells after ICSI (cleavage) and that formed a blastocyst at day 7 or 8 after ICSI. Cleavage and blastocyst rates of sperm injected oocytes from Young (n=29 oocytes from 13 mares) and Old (n=24 oocytes from 11 mares). Different superscripts indicate differences at $P < 0.05$ for the same end point (Fisher's exact test).

Discussion

We evaluated equine oocytes and early embryos to determine the effects of maternal aging on their metabolism, as a potential cause of the age-associated reduction in oocyte developmental potential. Our study describes novel findings using a noninvasive technology to assess metabolism in individual oocytes and embryos and describes metabolic alterations related to maternal aging. To the best of our knowledge, effects of maternal aging on individual oocyte and early-embryo metabolic function, as quantified by OCR and ECAR, have not been

demonstrated in any mammalian species. The present study indicates that equine maternal aging is associated with impaired oocyte metabolic function and capacity. Our findings suggest that not only do oocytes from old mares produce less energy under basal conditions, they are also not capable of as much energy output as oocytes from young mares. The cause and effects of altered oocyte metabolism in the old mare is still to be determined; however, the current study provides new insight into metabolic alterations associated with aging.

During follicular development and maturation, oocyte metabolism is highly dependent on the surrounding cumulus cells, which provide energy substrates to oocytes (Cecchino *et al.*, 2018). Glycolysis is a major pathway for production of energy in the follicle (Dumesic *et al.*, 2015). However, the oocyte has low capacity for glucose metabolism, thus cumulus cells actively uptake glucose from the follicular environment or culture media, metabolize it, and transport pyruvate and lactate to the oocyte via gap junctions (Su *et al.*, 2009; Wang *et al.*, 2012). Metabolite analyses of MII oocytes and their respective cumulus cells demonstrated no effect of maternal age on the relative abundance of glucose, pyruvate and lactate.

Oocytes also metabolize fatty acids for energy production through β-oxidation in mitochondria. This pathway is more important in species that have abundant lipids in their oocytes, such as bovine and porcine (Sturmey *et al.*, 2009; Paczkowski *et al.*, 2013). Likewise, equine oocytes have a large accumulation of lipids and use fatty acid metabolism during maturation (Pirro *et al.*, 2014), suggesting a reliance on β-oxidation to meet energy demands. In the present study, the relative abundance of FFA did not significantly differ in cumulus cells from young and old mares; conversely, FFA was lower in old than young mare oocytes. Potentially, the transport of FFA from cumulus cells to the oocyte is impaired with maternal aging. Although larger molecules than carbohydrates, FFA are also transported from the cumulus

cells to the oocyte through transzonal projections (TZPs) which extend through the zona pellucida (del Collado *et al.*, 2017). These cellular connections typically remain intact until the final stages of meiotic maturation (Barrett & Albertini, 2010; Clarke, 2018). However, a reduction in TZPs occurs with maternal aging in mice (El-Hayek *et al.*, 2018). While the abundance of TZPs has not been assessed in equine COCs, alterations in oocyte morphology in old mares are suggestive of an earlier breakdown of TZPs during maturation. Oocytes from old mares can have larger inner zona pellucida (ZP) and perivitelline space volumes despite similar ooplasm diameters (Altermatt *et al.*, 2009). This suggests that oocytes from old mares may grow larger and then shrink from the ZP, potentially disrupting TZPs. Fatty acids in the oocyte also come from the breakdown of TG molecules stored in lipid droplets, which are a major source of energy reserve for early embryonic development (Sturmey *et al.*, 2009; Dumesic *et al.*, 2015). During maturation, lipid metabolism is stimulated in COCs and is beneficial to oocyte developmental competence (Dunning *et al.*, 2014). Differences in the relative abundance of FFA in oocytes could also indicate reduced activity of lipases in COCs from old mares, which are needed to break down the TG into FFA. Lipase expression and activity were not assessed in this study. The proportion of different FFAs was similar among oocytes and cumulus cells from both age groups. These same FFAs, in different proportional contributions, have been reported to be major components in oocytes from other species, such as porcine, bovine and human (Dunning *et al.*, 2014).

Aerobic production of energy by the oocyte is essential for the completion of important events associated with maturation and fertilization. One of the most energy intensive activities within the oocyte is the assembly and disassembly of microtubules for spindle formation during meiosis (Chappel, 2013). Spindle abnormalities during maturation are more frequent in oocytes

from old than young mares (Carnevale *et al.*, 2012; Rizzo *et al.*, 2018, 2019). Energy production potential in individual oocytes is typically deduced from indirect static outcomes or group assays of pooled samples. In the present study, we were able to assess indices of aerobic and anaerobic energy production in individual oocytes and early embryos from young and old mares. We observed a reduction in the energy-producing capacity of mitochondria in oocytes from old mares, demonstrated by lower basal and maximal OCR compared to oocytes from young mares. These findings are in agreement with previous findings in oocytes associated with advanced female age that are suggestive of mitochondrial dysfunction, such as reduced ATP content (Iwata *et al.*, 2011; Simsek-Duran *et al.*, 2013), loss of mitochondria cristae and matrix density (Kushnir *et al.*, 2012; Rambags *et al.*, 2014), reduced mitochondrial membrane potential (Pasquariello *et al.*, 2019), and altered mtDNA copy numbers (Rambags *et al.*, 2006, 2014; Campos-Chillon *et al.*, 2015; Pasquariello *et al.*, 2019). In a recent study, it was reported that most of the glucose metabolism in equine COCs results in the production of lactate, while the majority of ATP production is from fatty acid oxidation supported by the ooplasmic lipid reserve (Lewis *et al.*, 2020). Therefore, the lower OCR observed in oocytes from old mares in the present study could reflect insufficient availability of FFA to meet ATP demands through oxidative metabolism.

Multiple events during oocyte cytoplasmic and nuclear maturation and embryonic development after fertilization are associated with anaerobic metabolism (Lamas-Toranzo *et al.*, 2018). Advanced maternal age also impaired oocyte anaerobic glycolytic activity and capacity, which may be attributed to loss of enzymatic activity, since glucose and pyruvate availability were similar in oocytes from both age groups. Therefore, oocytes from old mares did not compensate for the lower energy production from aerobic metabolism by increasing glycolysis, likely reflecting an overall reduction in their ability to produce energy for critical events. Indeed,

the ratio between basal ECAR and OCR was similar between the two groups, suggesting that maternal aging does not affect the relative contribution of glycolysis and aerobic metabolism in oocytes.

Cell cleavage after ICSI is another energy requiring event and reflects the ability of the oocyte to process the injected sperm and begin embryonic formation (Altermatt *et al.*, 2009). In agreement with oocyte findings, advanced mare age was associated with impaired mitochondrial function in zygotes that successfully cleaved into two or more cells after ICSI. The ratio of ECAR to OCR was not different between groups and similar to the ratio observed for oocytes, suggesting proportional contribution of aerobic and anaerobic metabolism in MII oocytes and early-stage embryos. Cumulus expansion during oocyte maturation represents the end of cumulus cell and oocyte metabolic cooperativity (Collado-Fernandez *et al.*, 2012). After that point, oocytes and early-stage embryos rely on energy produced from internal reserves, such as carboxylic acids (Sutton-McDowall & Thompson, 2015) and fatty acids (Krisher, 2013), as well as substrates obtained from culture media. The reduced aerobic metabolism in embryos derived from old mare oocytes is likely due to impaired mitochondrial capacity, and potentially limited availability of substrates, such as FFA, for oxidative metabolism during this critical time of growth. Other factors, such as enzymatic control of metabolic pathways and cofactors may also be involved, although they were not assessed in the present study.

Maximal oocyte OCR, stimulated by CCCP titrations, is indicative of cellular respiratory capacity and was consistently higher than basal OCR, demonstrating mitochondrial reserve capacity in MII oocytes. Such excess metabolic capacity is seemingly related to the increase in mtDNA copy numbers during oocyte maturation (Hendriks *et al.*, 2015; Lamas-Toranzo *et al.*, 2018) and the cytoplasmic bioenergetic capacity to maintain further embryonic development

(Van Blerkom, 2011). Day-2 embryos expressed significantly higher basal OCR and ECAR when compared to basal and maximal values from MII oocytes. When compared to mature oocytes, OCR increases as soon as 9 h after in vitro fertilization for bovine zygotes (Muller *et al.*, 2019) and at the 3- to 4-cell stage for human embryos (Hashimoto *et al.*, 2017). The finding that both OCR and ECAR increased from mature oocytes to cleavage-stage embryos implies that general metabolic function increases after fertilization. This increase may result, in part, from changes in mitochondrial distribution and morphology or from the recruitment of quiescent mitochondria into active states during early embryogenesis, as described in other species (Bavister & Squirrell, 2000; Bentov *et al.*, 2011; Harvey *et al.*, 2011; Van Blerkom, 2011). An increase in metabolism during the early stages of development is not caused by mitochondrial replication, as this begins at the blastocyst stage in equine embryos (Hendriks *et al.*, 2019).

In our study, we assessed mtDNA numbers to determine if this measurement, often used to estimate mitochondrial numbers, was directly associated with mitochondrial functional assays. In previous studies, equine oocyte mtDNA copy numbers were lower from older when compared to younger mares when assessing oocytes matured in vitro (Rambags *et al.*, 2014). During the first 12 hours of oocyte maturation in vivo, mtDNA copy numbers did not differ over time in young mare oocytes, although a linear decline occurred over time in old mares' oocytes (Campos-Chillon *et al.*, 2015). We found no age difference in mtDNA copy number in MII equine oocytes, and mtDNA was not indicative of mitochondrial function or capacity. Therefore, the lower respiratory capacity of oocytes from old mares in our study may be related to damaged or less active or dysfunctional mitochondria when compared to those in young mare oocytes, rather than reduced overall numbers of mitochondria. Altered mitochondrial morphology, mitochondrial swelling and loss of cristae were more frequently observed in oocytes collected

from ovaries harvested from slaughtered older than younger mares after maturation in vitro (Rambags *et al.*, 2014), suggesting that decreases in mitochondrial quality might have contributed to lower OCR in old versus young oocytes. Our results are consistent with a recent study in women that reported negative effects of maternal aging on morulae OCR but not on mtDNA copy numbers (Morimoto *et al.,* 2020).

Mare age did not alter cleavage rates after ICSI, in consonance with previous reports (Altermatt *et al.*, 2009; Frank *et al.*, 2019); however, blastocyst formation at day 7 or 8 after sperm injection was negatively affected by maternal aging. For human embryos, OCR at cleavage stages correlates with viability (Tejera *et al.*, 2012), a finding in agreement with our day-2 embryos. As energy demand and reliance on aerobic metabolism increase during embryonic development (Lane *et al.*, 2001; Gardner & Harvey, 2015; Sanchez *et al.*, 2019), lower blastocyst rates obtained from old mares might be related to reduced metabolic capacity and limited availability of energy substrates originating from their oocytes. Insufficient anaerobic metabolic capacity may also be involved, as oocytes and early embryos from old mares did not compensate for reduced mitochondrial metabolism by increasing anaerobic metabolism.

In conclusion, the metabolic activity and capacity of equine oocytes were impaired by maternal aging. Aerobic metabolism of equine oocytes presumably relies mainly on oxidation of FFA, which were less abundant in oocytes from old than young mares. Insufficient substrates might represent one reason for reduced mitochondrial function and capacity in oocytes from old versus young mares. Oocytes from old mares had limited aerobic and anaerobic metabolism under basal and stimulated conditions, and age-associated alterations were also observed in the metabolic function of ICSI-produced early embryos, with reduced basal aerobic metabolism.

These metabolic alterations may contribute to impaired developmental potential for oocytes from old mares, as observed by their failure to consistently reach the blastocyst stage of development.

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CHAPTER III: OOCYTE METABOLIC FUNCTION, LIPID COMPOSITION, AND DEVELOPMENTAL POTENTIAL ARE ALTERED BY DIET IN OLDER MARES[2]

Summary

Dietary supplementation is the most feasible method to improve oocyte function and developmental potential *in vivo*. During three experiments, oocytes were collected from maturing, dominant follicles of older mares to determine if short-term dietary supplements can alter oocyte metabolic function, lipid composition, and developmental potential. Over approximately 8 weeks, control mares were fed hay (CON) or hay and grain products (COB). Treated mares received supplements designed for equine wellness and gastrointestinal health, flaxseed oil, and a proprietary blend of fatty acid and antioxidant support (RSS) intended to increase antioxidant activity and lipid oxidation. RSS was modified for individual experiments with additional antioxidants or altered concentrations of n-3 to n-6 fatty acids. Oocytes from mares supplemented with RSS when compared to COB had higher basal oxygen consumption, indicative of higher aerobic metabolism, and proportionately more aerobic to anaerobic metabolism. In a second experiment, oocytes collected from the same mares prior to (CON) and after approximately 8 weeks of RSS supplementation had significantly reduced oocyte lipid abundance. In the final experiment, COB was compared to RSS supplementation, including RSS modified to proportionately reduce n-3 fatty acids and increase n-6 fatty acids. The ability

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of sperm-injected oocytes to develop into blastocysts was higher for RSS, regardless of fatty acid content, than for COB. We demonstrated that short-term diet supplementation can directly affect oocyte function in older mares, resulting in oocytes with increased metabolic activity, reduced lipid content, and increased developmental potential.

Keywords: Equine, oocyte, diet, aging, metabolism, follicle, embryo, granulosa cell

Introduction

Oocyte viability is essential for female fertility. Maternal factors can affect oocyte quality, potentially by causing alterations in oocyte metabolism (Babayev and Seli, 2015). Substrate preferences, lipid content, and metabolism vary among species, with oocyte lipid content affecting its reliance on the oxidation of carbohydrates or fatty acids (Dunning *et al.*, 2014; Dalbies-Tran *et al.*, 2020). Energy needed for oocyte development and maturation is primarily produced through aerobic mitochondrial metabolism (Ben-Meir *et al.*, 2015; Cecchino *et al.*, 2018). Mitochondria do not replicate until after blastocyst formation in several species, including the horse and human; therefore, mitochondria within the oocyte are responsible for providing energy during early embryo development (May-Panloup *et al.*, 2007; Spikings *et al.*, 2007; Wai *et al.*, 2010; Hashimoto *et al.*, 2017; Hendriks *et al.*, 2019).

After the initiation of antral formation, approximately 2 months are required for growth of the human oocyte and development of the follicle to the ovulatory stage (Williams and Erickson, 2012). The timeframe in the mare is likely similar, although not documented. Our ability to improve oocyte quality *in vivo* is limited. However, specific treatments or nutraceuticals can be used to target the follicle and oocyte during this growth phase. Although studies have reported some success with dietary supplementation (Nehra *et al.*, 2012; Ben-Meir *et al.*, 2015), no specific recommendations are available for women undergoing assisted

reproductive technology (ART) procedures (Cecchino *et al.*, 2018; Gaskins and Chavarro, 2018), and less guidance is available for mares. Distribution of nutrients to the oocyte is complicated by dependence on the surrounding follicle (Richani *et al.*, 2021). Granulosa cells line the ovarian follicle and have an essential role in metabolism and transport of nutrients from systemic circulation to follicular fluid, providing a local environment for the developing oocyte (Siu and Cheng, 2013). Cumulus cells, which surround the oocyte, acquire and channel nutrients to the oocyte through cellular projections (Richani *et al.*, 2021), convert energy forms for the oocyte, and potentially protect the oocyte from high levels of lipids (Aardema *et al.*, 2013; Lolicato *et al.*, 2015). Granulosa and cumulus cell metabolism is directly associated with oocyte metabolism (Cecchino *et al.*, 2018). In women, granulosa cell oxidative stress is related to impaired oocyte quality and developmental potential (Jančar *et al.*, 2007; Karuputhula *et al.*, 2013). The development of microsensors that can measure real-time fluctuations in oxygen and pH allows us the ability to deduce single-oocyte aerobic and anaerobic metabolism and quantify them as oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), respectively (Obeidat *et al.*, 2018, 2019). Utilizing this technology, we recently confirmed less efficient aerobic and anaerobic metabolism in oocytes collected from the dominant, maturing follicles of old compared to young mares, and impaired oocyte metabolism was associated with a significant reduction in developmental potential (Catandi *et al.*, 2019, 2021). The effect of diet on metabolism of individual oocytes has not been studied.

Dietary supplementation of antioxidants and polyunsaturated fatty acids (PUFAs), specifically long-chain omega-3 (n-3) fatty acids, have been studied in some species; but minimal information is available for the horse. Dietary antioxidants could counteract the effects of oxidative stress, and they have been associated with improved fertility in mice (Ben-Meir *et*

al., 2015; Meldrum *et al.*, 2016). The positive and negative effects of dietary n-3 PUFAs on female reproductive outcomes have been disputed (Gaskins and Chavarro, 2018; Zarezadeh *et al.*, 2019). The simplest primary form of dietary n-3 PUFA found in vegetable oils is α-linolenic acid (ALA, C18:3), which is an essential FA that can be converted to other long-chain, n-3 PUFAs including eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6) through desaturation and elongation reactions (Das, 2006). However, these reactions can be inefficient with competitive inhibition of the rate-limiting enzymes, delta-6-desaturases; this can occur with high dietary omega-6 (n-6) PUFAs, such as linoleic acid (LA;C18:2) (Zarezadeh *et al.*, 2019). The natural equine diet is based on grazing and is composed of approximately 83% n-3 PUFA and 17% n-6 PUFA present in oils derived from leaves (Hallebeek and Beynen, 2002; Frape, 2004). Such PUFA composition is similar to the Mediterranean human diet, which is characterized by a higher ratio of n-3 to n-6 PUFA, with the ingestion of less carbohydrate-rich foods and more fruits and vegetables (de Lorgeril and Salen, 2012; Broughton and Moley, 2017) and improved embryo quality for patients undergoing ART procedures (Kermack *et al.*, 2020). The modern equine diet commonly includes grain supplementation. Feeding a hay-based diet with 3 kg of cereal-based concentrate results in an inversion of fatty acid content to approximately 5% n-3 PUFAs and 95% n-6 PUFAs (Hallebeek and Beynen, 2002). These findings are consistent with the Western human diet characterized by high intake of cereal grains and more n-6 relative to n-3 PUFAs (Nehra *et al.*, 2012; Hess and Ross-Jones, 2014; Dhungana *et al.*, 2016). The extent that absolute versus relative dietary content of n-3 and n-6 PUFAs affect fertility is yet to be determined. Diets considered “healthy” (high consumption of vegetables, fruits, nuts and meat) versus “unhealthy” (high intake of solid oil, processed and junk food) resulted in the recovery of more MII oocytes and higher chances of pregnancy after ART in

women (Jahangirifar *et al.*, 2019). Diet effects are likely systemic and multifactorial, with overall diet or complex supplementation having a greater impact on reproductive outcomes than individual nutrients.

In the present study, we used older mares to examine the hypothesis that dietary supplements fed for a limited time would affect oocyte metabolic function, lipid composition, and developmental potential. More specifically, we determined if supplementation of compounds designed to promote overall wellness and cellular health would alter the follicle and oocyte destined for ovulation. We further elucidated the extent that altering antioxidants or n-3 versus n-6 PUFA in supplements would affect the oocyte.

Materials and Methods

Animals, diet supplementation, and experimental designs

Colorado State University's Institutional Animal Care and Use Committee approved all the procedures performed in this study. Three experiments were performed with similar groups of nonlactating mares of light-horse breeds during three consecutive breeding seasons. Some mares were used during multiple seasons. For all experiments, groups were housed in adjacent dry lots with sheds, mineral blocks, and water *ad libitum*; grass/alfalfa mix hay obtained from the same source in different years was fed at approximately 2% body weight daily. Nutritional and mycotoxin analyses of representative hay samples were performed and resulted in 14% crude protein, 1.8% crude fat, 14.5% crude fiber and were mycotoxin free. Diet additions were fed daily in the morning in individual pens to assure consumption.

Dietary supplements for the experiments were obtained from the same source (Platinum Performance, Inc., Buellton, CA). All treatment groups received supplements designed to

support equine wellness and gastrointestinal health [GI, Platinum Performance® GI (147 g), a combination of vitamins, trace minerals, amino acids, antioxidants, n-3 PUFA, probiotics, and prebiotics] and a proprietary blend of fatty acid and antioxidant support (Reproductive Support Supplement, RSS) that was modified for individual experiments (Table 1). Grain and pelleted feed were manufactured by one source (Nutrena®, Cargill, Inc., Minneapolis, MN) and purchased from local sources.

Three experiments were performed to evaluate the effect of feeding supplements to support reproduction in older mares, with some variation in treatment groups to test different types of compounds and reproductive endpoints. In the first experiment, oocyte metabolic function was assessed after feeding dietary supplement with additional antioxidants. In a second experiment, oocyte lipid profiles were assessed before and after feeding the diet supplement. In the third experiment, oocyte developmental potential was compared after supplementation with and without altering the relative abundance of omega-3 versus omega-6 PUFAs. The experimental design for each experiment is summarized in Figure 7.

In Experiment 1 (metabolic function), mares were provided group-specific feeding regimes for 8 to 13 weeks before samples were collected in July and August to assess granulosa cell, oocyte and early-embryo metabolic function. Twenty mares between 13 and 23 years (mean age of 18.5 years) and 485 and 670 kg body weight (BW) were paired by age and body type. One mare from each pair was randomly assigned to one of two groups, with the other mare assigned to the remaining group. Although each group contained one 13-year old mare, the remaining mares were older (≥ 17 years). The control group (COB, mean age of 18.6 years) received 450 g of a mix of corn, oats, and barley (Nutrena® C.O.B.,) topped with 60 mL of corn oil (Mazola®, ACH Food Companies, Inc., Memphis, TN), representing approximately 10% of the daily caloric

Table 1: Experiments 1 to 3 with groups, dietary components, daily amount fed to mares, and basic experimental designs.

Exp	Group	Dietary Component	Amount	Design
Exp 1: Metabolic function	COB	Corn, oat, barley blend[3] Corn oil	450 g 60 mL	Two groups of mares were fed a control diet (COB) or supplemented diet (RSS1) for approximately 8 weeks prior to sample collection.
	RSS1	GI supplement[4] Flaxseed oil[5] Repro Support Supplement[6] Pterostilbene Coenzyme Q10 Pyrroloquinoline quinone	147 g 60 mL 25.5 g 500 mg 500 mg 40 mg	
Exp 2: Lipid profiles	CON	*Pre-treatment sample*	0	Samples were collected from single group of mares prior to any diet supplementation (control, CON) and after 8 weeks of feeding RSS2.
	RSS2	*Post-treatment sample* GI supplement Flaxseed oil Repro Support Supplement SafeChoice (pelleted feed) Grain mix with molasses	 147 g 60 mL 51 g 680 g 227 g	
Contemp. Samples	CONQ	*Pre-treatment sample*	0	Contemporary samples to Exp 2 were collected from four mares prior to and after 8 weeks of CoQ. Samples were assessed for an effect of time; no direct comparisons were made.
	CoQ	*Post-treatment sample* Coenzyme Q10	 500 mg	
Exp 3[8]: Develop-mental potential	COB	Same as COB, Exp 1		Three groups of mares were fed a control diet (COB), RSS3 (same as RSS2) or RSS3M in which RSS3 was modified by reducing n-3 fatty acids and increasing n-6 fatty acids.
	RSS3	Same as RSS2 without pelleted feed and grain mix		
	RSS3M	GI supplement Corn oil Repro Support Supplement without DHA[7]	147 g 60 mL 50 g	

[3] Provided 5.04g linoleic acid (LA) and 0.19 g alpha-linolenic acid (ALA)
[4] Platinum Performance® GI, Platinum Performance Inc., Buellton, CA
[5] Healthy Weight, Platinum Performance Inc. Provided 8.46g LA, 30.8g ALA and 480 IU d-Alpha Tocopherol Acetate
[6] Reproductive Support Supplement Proprietary Blend, Platinum Performance Inc. Provided ALA, docosahexaenoic acid (DHA), ascorbic acid, acetyl-L-carnitine, L-carnitine tartrate, and d-alpha tocopherol acetate
[7] Proprietary Blend, Platinum Performance Inc. Provided ALA, ascorbic acid, acetyl-L-carnitine, L-carnitine tartrate, and d-alpha tocopherol acetate
[8] Oocytes collected during Experient 1 (COB and RSS1) were also used to assess oocyte developmental potential in Experiment 3

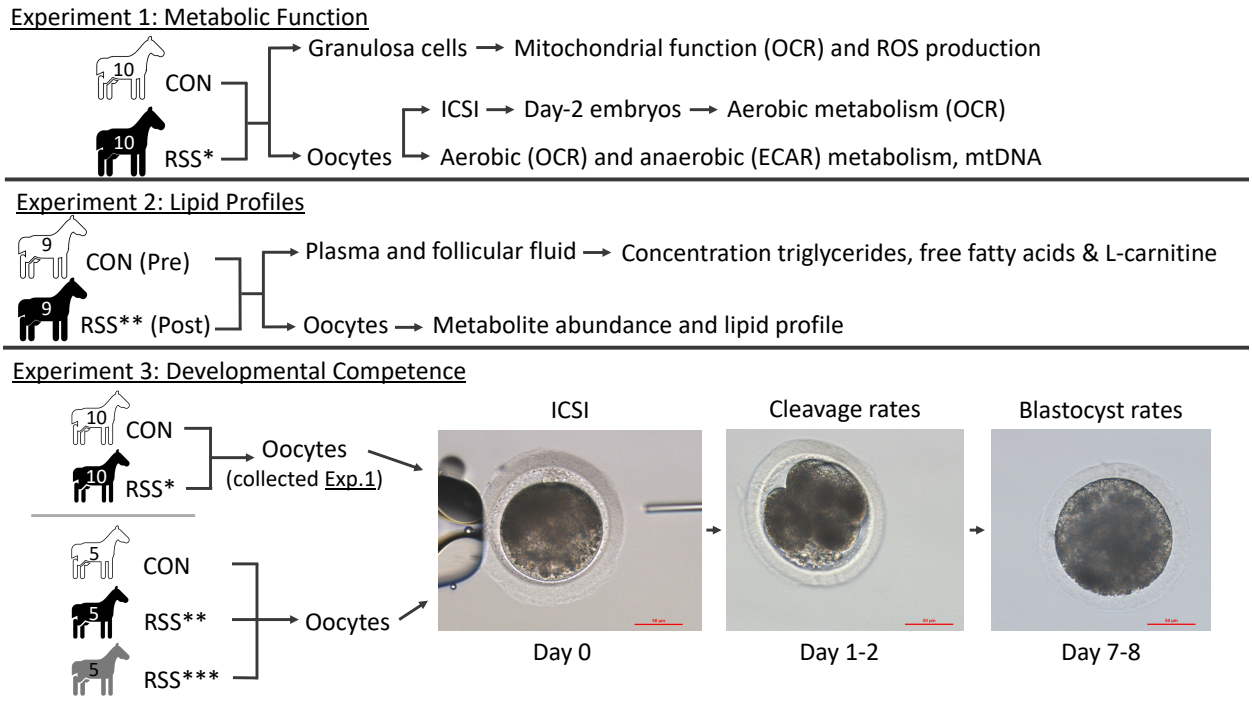

Figure 7: General experimental design. Control (CON) diets included only hay (Experiment 2) or hay and grain products (Experiments 1 and 3). Reproductive Support Supplements (RSS*, RSS** and RSS***) differed as described in Table 1. Samples were collected after ≥ 8 weeks of supplementation of matched groups of mares in Experiments 1 and 3. In Experiment 2, samples were collected from the same mares pre (CON) and post supplementation (RSS**). Representative images of an oocyte, early embryo (Day 2), and blastocyst are shown from a treatment mare (RSS**). Number of mares per group are shown within the horse icons.

intake. The relative n-6 to n-3 PUFA ratio of the COB supplementations was approximately 42:1. The treatment group of mares (RSS1, mean age of 18.5 years) was fed approximately equicaloric commercial supplements including GI and RSS at 25.5 g daily (Table 1). In addition, a flaxseed and natural vitamin E (d-alpha tocopherol acetate) oil [Healthy Weight Oil (60 mL)] supplied additional n-3 PUFA, providing a relative n-6 to n-3 PUFA ratio of 0.3:1. Specific antioxidants (coenzyme Q10, 500 mg; pterostilbene, 500 mg; pyrroloquinoline quinone, 40 mg) were also provided to the treatment group (RSS1, Table 1).

For Experiment 2 (lipid profiles), oocytes, follicular fluid, and blood were collected from mares in May or June (pre-treatment). The mares were then fed supplements for 8 to 10 weeks

before post-treatment samples from the same mares were collected in August. Oocyte lipid composition in addition to follicular and systemic concentrations of lipids and L-carnitine were determined pre- and post-supplementation from nine mares (16 to 22 years, mean age of 18.7 years, and 472 and 577 kg BW). For this experiment, mares were supplemented daily with GI, RSS (51g) and 60 mL of flaxseed oil (RSS2, Table 1). The supplements were mixed with a pelleted complete feed (Nutrena® SafeChoice Original, 14% crude protein, 7% crude fat, 680 g) and a mixed grain blend with molasses (Nutrena® Rocky Mountain Sweet Feed, 8% protein and 2% fat, 227 g) to increase palatability.

During the same period of time, a contemporary group of four mares (16 to 22 years, mean age of 18.7 years, 453 to 568 kg BW) were maintained on the same hay diet and in the same housing conditions as mares in Experiment 2, but they were supplemented only with an antioxidant (coenzyme Q10, 500 mg daily) and no other dietary additives (Table 1). Oocyte metabolite content was also assessed for these mares to determine if oocyte composition was altered over time in mares not given the reproductive support supplement. No direct statistical comparisons were made between the treated mares in Experiment 2 and in this contemporary group of mares.

For Experiment 3 (developmental competence), oocytes were obtained from the mares in Experiment 1 or were obtained from additional groups of mares fed varying proportions of n-3 and n-6 PUFAs. For these mares, supplements were provided for 8 to 17 weeks before oocytes were collected and injected with sperm to assess developmental potential. Fifteen mares aged 18 to 24 years (mean age of 20.6 years, 440 to 610 kg BW) were grouped by age and then randomly assigned to one of three groups. The control group of mares (COB, n=5, mean age of 20.4 years) received corn, oats and barley and corn oil supplements (Table 1). A second group of mares

(n=5, mean age of 20.4 years) received RSS3 (same supplements as RSS2 in Experiment 2), with a relative n-6 to n-3 PUFA ratio of 0.3:1. For the third group of mares (RSS3M, n=5, mean age of 20.8 years), the supplements were modified by the removal of DHA from RSS and flaxseed oil was replaced with corn oil, reducing n-3 PUFAs and increasing n-6 PUFAs in the diet to a n-6 to n-3 PUFA ratio of 2.1:1 (Table 1).

Oocyte collection and maturation

Oocytes were collected from dominant, follicular-phase follicles to provide consistency in the stage of development and evaluation of oocytes which were destined to ovulate naturally. Follicular maturation was induced during the follicular phase when a dominant follicle ≥ 35 mm in diameter and endometrial edema were observed using ultrasonography. Induction occurred from the administration of human chorionic gonadotropin (2000 IU, intravenous; Chorulon, Merck Animal Health, Madison, NJ) and deslorelin acetate in an aqueous base (0.75 mg, intramuscular; Precision Pharmacy, Bakersfield, CA) in Experiments 1 and 2 and histrelin in aqueous base (0.5 mg, intramuscular, Doc Lane, Lexington, KY) in Experiment 3. Cumulus oocyte complexes (COCs) were collected by transvaginal, ultrasound-guided follicular aspiration of dominant follicles at 16±2 h after induction in Experiment 1 and at 20±2 h in Experiments 2 and 3 as described previously (Carnevale, 2016). Recovered COCs were incubated in media [TCM199 with Earle's salts (GibcoTM, Thermo Fisher Scientific, Waltham, MA) with 10% fetal bovine serum (FBS), 25 μg/mL of gentamicin, and 0.2 mM pyruvate] at 38.2°C in an atmosphere of 5% CO_2 and air for 26±2 h in Experiment 1 and 22±2 h in Experiment 3. After maturation, oocytes were stripped of cumulus cells by sequential pipetting in a MOPS-buffered medium (G-MOPS, Vitrolife, Englewood, CO) with 0.04% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO) and hyaluronidase (200 IU/mL; Sigma-Aldrich). For electrochemical measurements

of basal and maximal oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) in Experiment 1, oocytes were moved to a MOPS-buffered medium (G-MOPS) with 0.04% BSA at 38.2°C until the assay. In Experiment 2, recovered oocytes were stripped of cumulus cells as described above, carefully evaluated to confirm complete removal of cumulus cells, fixed in 100 µL of 50% methanol solution, snap frozen in liquid nitrogen, and stored at -80°C until mass spectrometry analyses.

Granulosa cell collection, OCR, and reactive oxygen species (ROS) production assays

In Experiment 1, granulosa cells were collected at the time of oocyte recoveries. For mitochondrial OCR and ROS release data collection, granulosa cells (COB, n=7 and RSS1, n=5) were separated from the follicular aspirates, suspended in flush solution (Vigro Complete Flush Solution, Vetoquinol, Fort Worth, TX), and pelleted for further use. The flush solution supernatant was aspirated, and granulosa cells were resuspended and washed in mitochondrial respiration medium (MiR05) containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, and 110 mM D-sucrose. Cells were resuspended and washed twice by pulling 1000 µl of cells from the flush solution and placing them in a 2-mL Eppendorf tube containing 1000 µl of MiR05. Cells were then mechanically washed by pipetting up and down before being pelleted and washed again in a separate Eppendorf tube containing 1000 µl of MiR05. The entire cell suspension was added to a 2-mL chamber in an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria). OCR was monitored in real-time by resolving changes in the negative time derivative of the chamber oxygen concentration signal. This signal was normalized to the protein concentration of the granulosa cell pellet that was collected at the end of experiments. Respirometry chambers were maintained at 37 °C under atmospheric oxygen concentration (100-

200 μM O_2) to avoid potential limitations in oxygen diffusion on respiratory capacity. Basal OCR (respiration of intact cells supported by endogenous substrates) was measured prior to permeabilization of cell membrane with digitonin (15 μg/mL) to provide mitochondrial access to cell-impermeable substrates. Mitochondrial respiratory flux and maximal OCR were stimulated by the addition of multiple substrates in the following sequence: 1 mM malate, 5 mM pyruvate, 2.5 mM ADP, 10 mM glutamate, and 10 mM succinate as previously described (Chicco *et al.*, 2018). For determination of ROS release, 10 μM Amplex Red and horseradish peroxidase, at a final concentration of 1 U/mL, were added to the oxygraph chamber after the addition of granulosa cells for fluorescence measurement. Horseradish peroxidase combines with hydrogen peroxide (H_2O_2, a membrane permeable ROS species) and irreversibly oxidizes Amplex Red to resorufin (Ex/Em 571/585 nm) while reducing H_2O_2 to two equivalents of H_2O (Goo *et al.*, 2013). ROS data are presented as the rate of release per second.

In vitro embryo production

Frozen-thawed semen from a single ejaculate from one stallion was used for sperm injections. Approximately one-tenth of a 0.25-mL straw of frozen semen was cut under liquid nitrogen and thawed directly in 1 mL of MOPS-buffered medium (G-MOPS) with 0.04% BSA at 38.2°C; one sperm was selected and prepared for sperm injection as previously described (Gonzalez-Castro and Carnevale, 2018). Intracytoplasmic sperm injection (ICSI) was performed in a MOPS-buffered medium (G-MOPS) with 0.04% BSA using a micromanipulator (Narishige Group, Amityville, NY) and a piezo-driven injection system (Prime Tech, Japan). After sperm injection, presumptive zygotes were placed into an embryo culture medium (global®, LifeGlobal Group, Guilford, CT) with 10% FBS in individual 30-μL droplets under paraffin oil (OVOIL™, Vitrolife) and incubated at 38.2°C in 5% O_2, 7% CO_2, and 88% N_2.

In Experiment 1, at 2 days after ICSI (46-57 h, mean of 52.2 h), early embryos with normal morphology and 2 to 8 cells (mean of 4 cells) were used for the measurement of basal OCR (COB, n=11 and RSS1, n=12). A set of early embryos were stimulated for maximal OCR (COB, n=6 and RSS1, n=6). Additional embryos were cultured in Experiments 1 (COB, n=13 and RSS1, n=12) and 3 (COB, n=19; RSS3, n=15; and RSS3M, n=24) to determine blastocyst development rates. These embryos were moved at 5 days after ICSI into individual 30-µl droplets of a second culture medium (global® for fertilization, LifeGlobal Group) with 10% FBS. Embryos were observed daily until blastocyst formation or degeneration (Carnevale and Metcalf, 2019).

Oocyte and early embryo OCR and ECAR assays

Metabolic analyses of oocytes (COB, n=4 and RSS1, n=4) and early embryos in Experiment 1 were performed using a microchamber with electrochemical-based oxygen and pH sensors as described in detail (Obeidat *et al.*, 2019). Before each oocyte or early embryo assay, the microchamber was filled with 120 µL of MOPS-buffered medium (G-MOPS) with 0.04% BSA, overlaid with 120 µL of paraffin oil (OVOIL™) to limit the chamber from atmospheric oxygen diffusion. The three-electrode system for the oxygen sensor was connected to a potentiostat (Quadstat EA 164H, eDAQ Inc., Colorado Springs, CO) that applied -0.6 V to the sensor and monitored the decrease in oxygen reduction current over time. The pH sensor was connected to a custom-made Ina333 instrumentation amplifier circuit that measured the change in voltage. The starting oxygen concentration and pH of the medium were measured as baseline current. After that, individual oocytes or early embryos were transferred into the microchamber.

Calculations of sample OCR and ECAR were based on rates of change for oxygen and pH, respectively, from baseline values over time. Initially, basal OCR was assayed for 5 min,

followed by basal ECAR assessment for 2 min. Three titrations of 1 μM of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a mitochondria uncoupler that stimulates maximal oxygen consumption, were performed for oocyte and early embryos, with OCR measurements for 5 min after each CCCP addition. Maximal OCR was defined as the highest observed value during CCCP titrations. An additional measurement for oocyte ECAR was determined after the first addition of CCCP, as an indication of the oocyte's ability to use anaerobic pathways when mitochondrial energy production is limited. After metabolic readings, individual oocytes were stored at -80^{o}C until mtDNA quantification.

Oocyte mitochondrial DNA content absolute quantification

Mitochondrial DNA (mtDNA) content of single oocytes (COB, n=16 and RSS1, n=18) was quantified by real-time PCR (qPCR) as previously described (Pasquariello *et al.*, 2019; Catandi *et al.*, 2021). Kits and supplies from one source (Qiagen, Germantown, MD) were used unless noted. Briefly, DNA extraction of individual oocytes was performed using the QIAamp DNA micro kit according to the manufacturer's protocol with the addition of carrier RNA (1 μg) to each sample. The DNA sample was eluted with 50 μL of Buffer AE (supplied with the kit) and analyzed for qPCR using an absolute quantification assay. To this end, quantification standards were prepared; a 1096-bp fragment of the I-rRNA region of equine mtDNA was amplified by PCR using the LongRange PCR Kit and the primer pair 5'-AGCAATTTCGGTTGGGGTGA-3' and 5'-GCTCGGTTGGTTTCGGCTAA-3'. The fragment was then purified using the Qiaquick PCR purification kit and cloned using the Qiagen PCR cloning kit. Plasmid DNA containing the amplified mtDNA fragment was purified from bacteria using the Qiaprep miniprep kit. A standard curve was generated by using seven tenfold serial dilutions (10^7 to 10 copies), and standard curve correlation coefficients were greater than 0.98.

Real-time quantitative PCR using the primer pair 5'-ATGGTTTGTGCTACTGCTCG-3' and 5'-GCCCTAACCCTGGCCTTAAC-3' was run in triplicate for each standard dilution and sample in 10-µL reactions using PowerUp SYBR Green master mix (Applied Biosystems, Foster City, CA), a LightCycler 480II (Roche Applied Science, Indianapolis, IN). The program of amplification was: 50ºC for 2 min for the first cycle; 95ºC for 2 min for the second cycle; 95ºC for 15 s and 60ºC for 1 min for 40 cycles; a melting curve was run to assess specificity of the primers. Samples and standard curve were run on the same plate. Copy numbers of mtDNA in each oocyte were generated from the equation of Ct value against copy number for the corresponding standard curve.

Oocyte metabolomics analyses by liquid chromatography coupled to mass spectrometry

Metabolites in oocytes (RSS2, n=9 pre- and post-supplementation) were first extracted by adding 400 µL of 100% LCMS-grade methanol to each sample while still frozen. The samples were then shaken for 1 h at 4°C. The samples were sonicated in a cold bath for 30 min, shaken again for 1 h at 4°C, and sonicated in a cold bath for 30 min. The samples were centrifuged briefly, and all 500 µL of sample was dried completely with nitrogen and resuspended in 60 µL of 1:1 methanol/toluene. A quality control (QC) sample was pooled by taking 15 µL per sample.

Five microliters of extract were injected onto a ACQUITY UPLC system (Waters, Milford, MA) in randomized order with a QC injection after every 6 samples. Separation was achieved using a ACQUITY UPLC CSH Phenyl Hexyl column (1.7 µM, 1.0 x 100 mm) (Waters), using a gradient from solvent A (water, 2mM ammonium formate) to solvent B (acetonitrile, 0.1% formic acid). Injections were made in 99% A, held at 99% A for 1 min, ramped to 98% B over 12 min, held at 98% B for 3 min, and then returned to starting conditions over 0.05 min and allowed to re-equilibrate for 3.95 min, with a 200 µL/min constant flow rate.

The column and samples were held at 65 °C and 6 °C, respectively. The column eluent was infused into a Xevo G2-XS Q-TOF-MS (Waters) with an electrospray source in positive mode, scanning 50-1200 m/z at 0.2 s per scan, alternating between MS (6 V collision energy) and MSE mode (15-30 V ramp). Calibration was performed using sodium formate with 1 ppm mass accuracy. The capillary voltage was held at 700 V, source temperature at 140 °C, and nitrogen desolvation temperature at 600°C with a desolvation gas flow rate of 1000 L/h.

Raw mass spectrometry data were processed using an R-based workflow for feature detection, retention time alignment, feature grouping, peak filling, feature clustering. RAMClustR version 1.1.0 in R version 3.6.3 was used to normalize, filter, and group features into spectra. XCMS (Smith *et al.*, 2006; Tautenhahn *et al.*, 2008) output data was transferred to a ramclustR object using the rc.get.xcms.data function. Feature data was extracted using the xcms featureValues function; features that failed to demonstrate signal intensity of at least 3-fold greater in QC samples than in blanks were removed from the feature dataset (3642 of 24810 features were removed). Features with missing values were replaced with small values simulating noise. For each feature, the minimum detected value was multiplied by 0.1. Noise was then added using a factor of 0.1. The absolute value was used to fill the noise values to ensure that only non-negative values carried forward. Variance in quality control samples was described using the rc.qc function within ramclustR. Features were normalized by linear regression of run order versus qc feature intensities to account for instrument signal intensity drift. Only features with a regression p-values less than 0.05 and an r-squared greater than 0.1 were corrected. Features were additionally normalized to the total extracted ion signal to account for differences in total solute concentration. Normalized peak areas for individual metabolites were compared between pre- and post-supplementation paired samples; within each lipid category, individual

metabolites were summed to obtain a peak area for the total normalized abundance for each lipid category.

Follicular fluid and blood collection and analyses

In Experiment 2, plasma and follicular fluid were collected for triglyceride, fatty acid, and L-carnitine assays (RSS2, n=9 RSS2 pre- and post-supplementation). To prevent cellular and blood contamination of follicular fluid samples, the aspiration needle was not rinsed with media and was inserted into the central antrum of the follicle before approximately 5 mL of follicular fluid were gently aspirated into a collection tube. The follicular fluid was aliquoted and stored at -80^{o}C until assays. Blood was collected by jugular venipuncture prior to morning feeding. It was centrifuged at 200 x g for 10 min at room temperature; supernatant was recentrifuged at 1500 x g for 10 min at room temperature, then aliquoted and stored at -80^{o}C until assays.

Triglyceride concentrations in plasma and follicular fluid samples were determined using a colorimetric assay kit (Cayman Chemicals, Ann Harbor, MI) according to kit instructions. The 96-well, non-treated microplate (Thermo Fisher Scientific) was read at 540-nm absorbance on a Synergy 2 microplate reader (Biotek, Winooski, VT). All samples were assayed in a single plate. The intra-assay coefficient of variation was 1.35%, and the minimal detectable concentration was 1 mg/dl. Concentrations of free fatty acids in plasma and follicular fluid were determined with a colorimetric assay kit (Bioassay Systems, Hayward, CA) according to the manufacturer's instructions. The 96-well microplate was read at 570-nm absorbance using the same microplate reader. The intra-assay coefficient of variation was 3.84%, and the sensitivity was 7 uM. L-carnitine concentrations in plasma and follicular fluid were determined with a colorimetric assay kit (BioVision, Milpitas, CA) following the kit instructions. The 96-well microplate was read at

570-nm absorbance with the same equipment. The intra-assay coefficient of variation was 2.14%, and the limit of detection was 10 uM.

Statistical analyses

Statistical analyses were completed using GraphPad Prism 8.0.2 (GraphPad Software, Inc., San Diego, CA). Student's *t*-tests were used to compare continuous data in Experiment 1, Fisher's exact tests, and chi-square tests were used to compare cleavage and blastocyst rates in Experiments 1 and 3. In Experiment 2, ANOVA with Kenward-Roger degrees of freedom and false discovery rate adjustments were used to compare metabolite abundance in pre- and post-supplementation oocytes; paired t-tests and Wilcoxon tests were used to compare continuous data. Values of $P < 0.05$ were considered significant, and $P \leq 0.1$ was considered tending toward significance. Results are presented as mean ± SEM.

Results

Experiment 1: Effect of diet supplementation on cell metabolic function

Mares were supplemented with grain (COB, corn, oats and barley) or a reproductive support supplement with additional antioxidants (RSS1) prior to the assessment of aerobic metabolism, based on OCR, or anaerobic metabolism, based on ECAR, of granulosa cells, oocytes, and early cleavage embryos.

Supplementations did not significantly affect granulosa cell aerobic metabolism or ROS production (Figures 8A-E). However, the production of ROS relative to aerobic metabolism (ROS/OCR) was higher (P=0.03) for mares fed grain than the reproductive support supplement under substrate-stimulated respiration, indicating that a higher proportion of oxygen consumption resulted in ROS production than in the generation of ATP (Figure 8F).

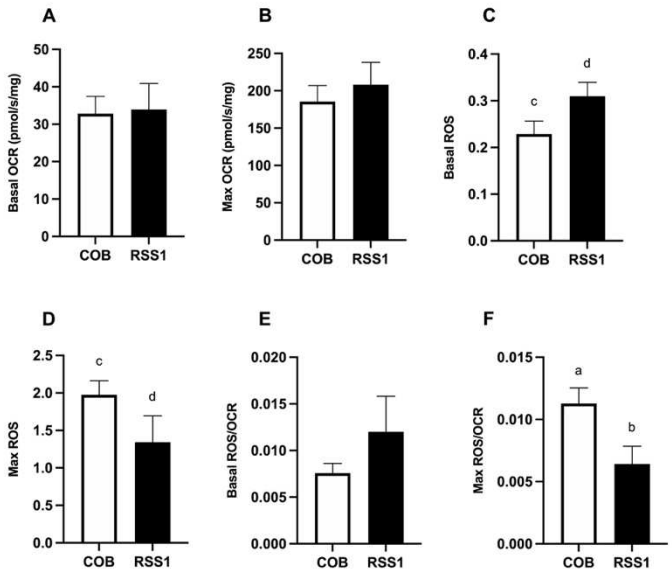

Figure 8: Granulosa cell aerobic metabolism, based on oxygen consumption rate (OCR), and production of reactive oxygen species (ROS) under basal and stimulated conditions from older mares supplemented with grain and corn oil (COB) or complex nutrients to support health and reproductive function (RSS1). (A) Basal OCR, (B) Maximal (stimulated) OCR, (C) ROS formation under basal conditions, (D) ROS formation under stimulated conditions, (E) proportion of basal OCR related to ROS production, (F) proportion of maximal OCR related to ROS production (COB, n=7; RSS1, n=5). Barcharts present means ± SEMs. Different superscripts indicate differences (ab, $P<0.05$) or a tendency to differ (cd, $P\leq0.1$). Supplement components are listed in Table 1.

Basal, but not maximal, aerobic metabolism was lower (P=0.008) for oocytes collected from mares fed grain than the reproductive support supplement (Figure 9A and B). Mitochondrial efficiency, representing the proportion of maximal cell respiratory capacity used during basal metabolism (basal OCR/maximal OCR) was lower (P=0.02) when mares were fed grain than the diet support supplement (Figure 9C). However, mitochondrial reserve capacity (maximal OCR – basal OCR), was similar (P=0.03) between the groups, suggesting that the oocytes were capable of similar responses to energy demands (Figure 9D). As aerobic respiration occurs in mitochondria, mitochondria DNA copy numbers were analyzed as an indicator of the number of mitochondria within oocytes. However, in contrast to metabolic activity, mtDNA

were higher (P=0.04) in oocytes from mares fed grain than the reproductive support supplement (Figure 9E).

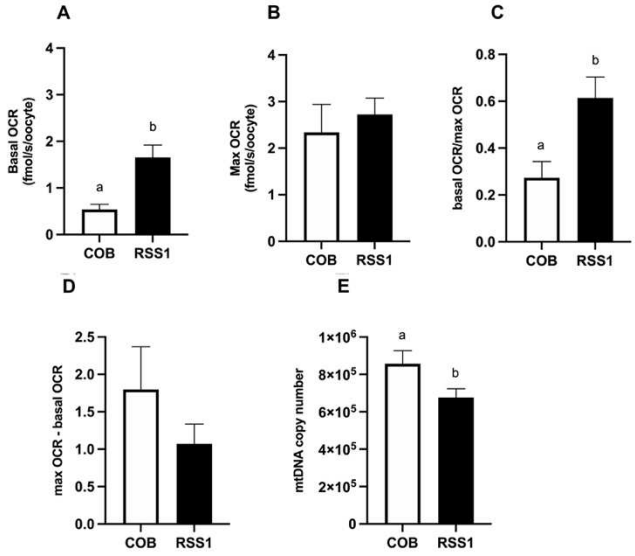

Figure 9: Aerobic metabolism, based on oxygen consumption rate (OCR), and DNA copy numbers in metaphase II oocytes from older mares supplemented with grain and corn oil (COB) or complex nutrients to support health and reproductive function (RSS1). (A) Basal OCR, (B) maximal OCR, (C) mitochondrial efficiency (basal OCR/max OCR), (D) mitochondrial reserve capacity (max OCR – basal OCR) (COB, n=4; RSS1, n=4) and (E) quantification of mtDNA copy number (COB, n=16; RSS1, n=18). Barcharts present means ± SEMs. Different superscripts indicate differences at P<0.05.

Oocyte basal anaerobic metabolism, based on the extracellular acidification rate (ECAR), did not differ between groups (Figure 10A); although when stimulated, maximal anaerobic metabolism was higher (P=0.04) for oocytes from mares fed the support supplement when compared to grains (Figure 10B). Oocytes from mares fed grains used proportionately more (P=0.006) anaerobic to aerobic metabolism (based on ratios of ECAR/OCR and reflecting the glycolytic rate to oxidative phosphorylation rate) (Figure 10C). No significant differences were observed for the metabolic activity of early embryos resulting from ICSI of oocytes from mares supplemented with grains or reproductive support supplement (Supplementary Figure 1A and B, Appendix II). In total, results of the first experiment demonstrate differences in mitochondrial

function in the oocytes from old mares fed grain products or provided nutrients for reproductive support.

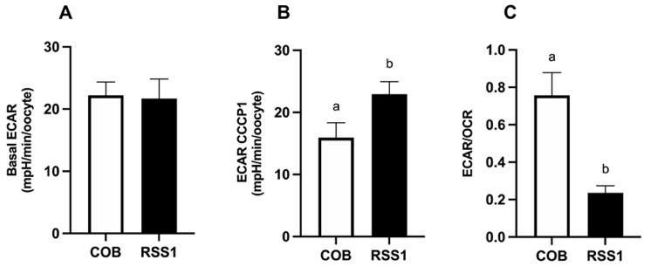

Figure 10: Anaerobic metabolism, based on extracellular acidification rate (ECAR), of metaphase II oocytes from older mares supplemented with grain and corn oil (COB) or complex nutrients to support health and reproductive function (RSS1). (A) Basal ECAR, (B) ECAR after first addition of CCCP (COB, n=9; RSS1, n=8) and (C) proportion of basal anaerobic to aerobic metabolism (COB, n=4; RSS1, n=4). Barcharts present mean ± SEM. Different superscripts indicate differences at $P<0.05$.

Experiment 2: Effects of diet supplement on systemic, follicular, and oocyte lipid concentrations

Oocyte metabolome and systemic and follicular concentrations of lipids were assessed for mares prior to and after being fed a reproductive support supplement (RSS2). A total of 1585 metabolites were assessed in oocytes; 441 metabolites differed (n=211, $p<0.05$) or tended to differ (n=230, $p\leq0.1$) in oocytes that were collected before when compared to after diet supplementation with most differences observed in lipids (n=802 total lipid metabolites, n=118, $p<0.05$ and n=139, $p\leq0.1$). Differences in lipid abundance were primarily noted for glycerolipids (Figure 11A), although generally species of lipids were consistently less abundant in oocytes collected after than before diet supplementation.

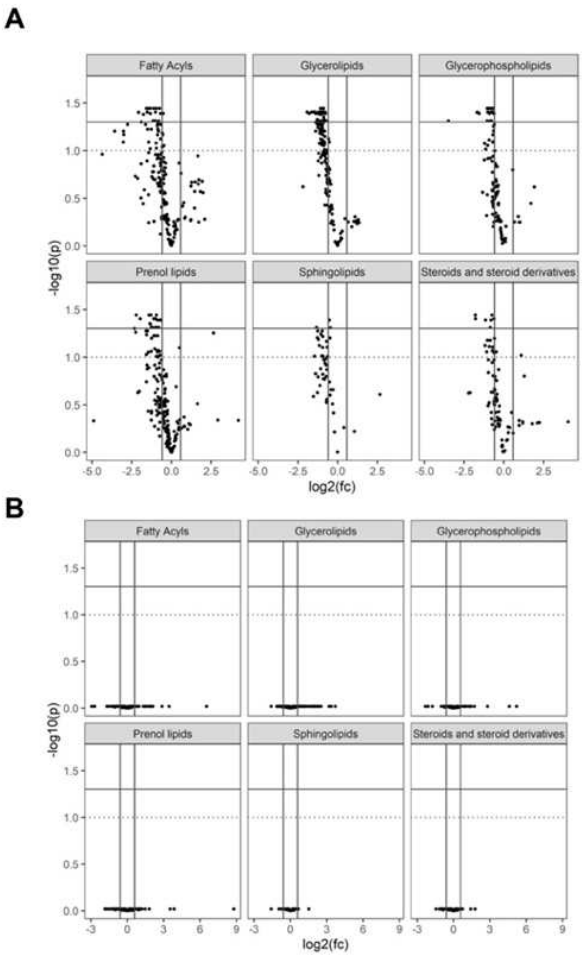

Figure 11: Volcano plots illustrating lipid categories in oocytes from older mares (A) pre- and post-supplementation with complex nutrients to support health and reproductive function (RSS2, n=9), and (B) pre- and post-supplementation with coenzyme Q10 (n=4). Negative log2(fc) indicates lipids that were reduced in oocytes post-diet supplementation, while positive log2(fc) indicates lipids that were elevated in post-diet supplementation oocytes. The horizontal bars indicate significance at P<0.05 (solid line) and P<0.1 (dotted line).

Normalized abundance of total triglycerides, glycerophospholipids, diacylglycerols, free fatty acids, sphingomyelins, cholesteryl esters, glycerophosphocholines, and glycerophosphoserines were significantly higher before when compared to after supplementation (Figures 12). In a contemporary group of mares consuming only coenzyme Q10 (CoQ) in addition to hay, lipid content and normalized abundance of lipid categories did not differ before or after feeding the antioxidant (CoQ) during the same time interval (Figures 11B and 12).

Results from this contemporary group of mares demonstrate that oocyte lipid content did not change over time when the reproductive support supplement was not fed. Therefore, dietary supplementations resulted in changes in oocyte composition, most notably in lipid abundance. Oocyte metabolites affected by the diet supplements are presented in Supplementary Tables 2 and 3 (Appendix II).

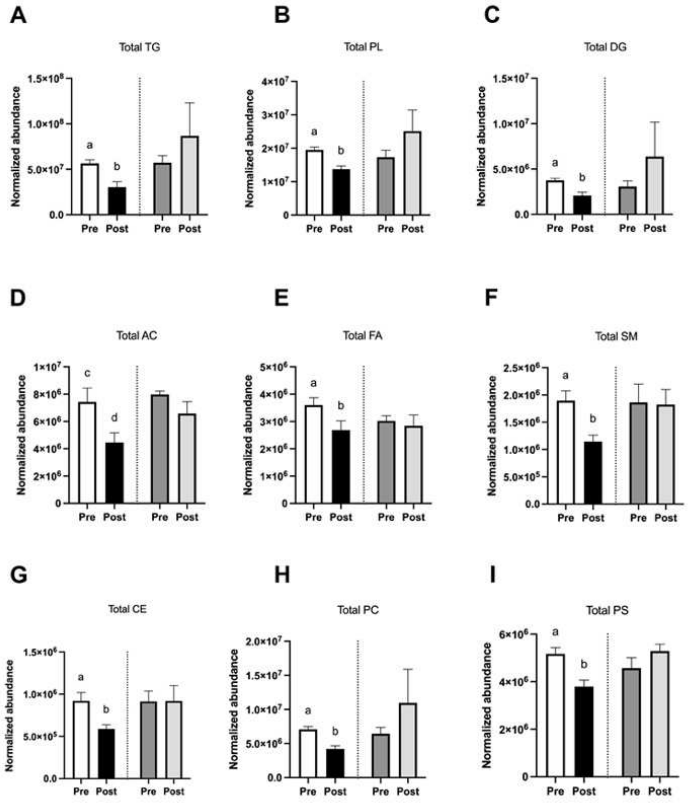

Figure 12: Normalized abundance of total (A) triglycerides, (B) glycerophospholipids, (C) diacylglycerols, (D) acylcarnitines, (E) free fatty acids, (F) sphingomyelins, (G) cholesteryl esters, (H) glycerophosphocholines, and (I) glycerophosphoserines in oocytes from older mares pre- (white bars) and post-supplementation with RSS2 (black bars; n=9) and pre- (dark grey bars) and post-supplementation with coenzyme Q10 (light grey bars; n=4). Barcharts represent means ± SEMs. Different superscript for pre- and post-supplementation with RSS2 indicate differences (ab, $P<0.05$ or cd, $P<0.1$). No significant differences were observed pre- and post-supplementation with coenzyme Q10.

Systemic and follicular fluid concentrations of lipids were assessed before and after feeding the diet supplement. Control (pre-supplement) concentrations of triglycerides and free

fatty acids were higher in plasma than in follicular fluid. Diet supplementation resulted in reduced ($P<0.01$) concentrations of follicular fluid triglycerides and plasma fatty acids (Figure 13A and B). Concentrations of L-carnitine in plasma and follicular fluid did not significantly differ with fluid type or diet supplementation (Figure 13C).

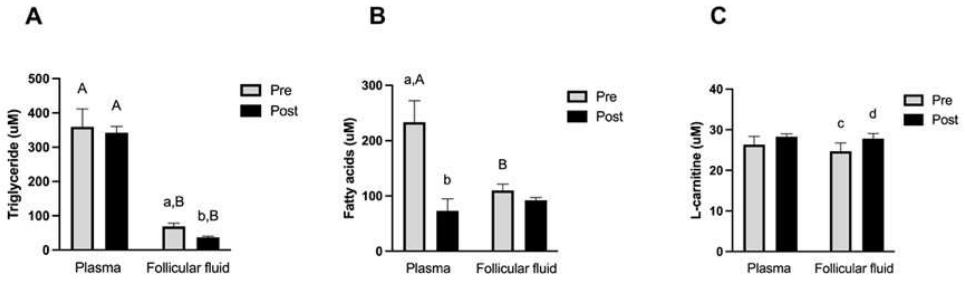

Figure 13: Concentrations of (A) triglycerides, (B) free fatty acids and (C) L-carnitine in plasma and follicular fluid from older mares (n=9) pre- and post-supplementation with complex nutrients to support health and reproductive function (RSS2). Barcharts represent means ± SEMs. Different superscripts between pre- and post-supplementation for plasma or for follicular fluid represent differences (ab, $P<0.05$ and cd, $P<0.1$); different superscripts for the same endpoint between plasma and follicular fluid indicate significance (AB, $P<0.05$).

Experiment 3: Effect of maternal diet on oocyte developmental potential

Oocyte developmental potential to the blastocyst stage was assessed between grain-fed control mares and mares fed a reproductive support supplement with additional antioxidants or with variable levels of omega-3 versus omega-6 fatty acids (see RSS for Experiments 1 and 3, Table 1). Cleavage rates of sperm-injected oocytes at 1 or 2 days after ICSI were similar for mares provided a grain supplement (12/13, 92%) or the reproductive support supplement with additional antioxidants (11/12, 92%) (Figure 14A); however, more blastocysts developed per sperm-injected oocytes by day 7 or 8 after ICSI for mares supplemented with the reproductive support supplement than with grains (7/12, 58% and 2/13, 15%, respectively, $P=0.04$) (Figure 14A). When fatty acid concentrations were varied, cleavage rates were not significantly different among groups (Figure 14B). The number of blastocysts per injected oocyte were similar for

oocytes from mares fed the reproductive support supplements, regardless of omega-3 and omega-6 fatty acid content, but higher (P≤0.02) than for mares supplemented with grain (supplement with omega-3 fatty acids, 6/15, 40%; supplement with substitution of n-3 with n-6 fatty acids, 10/24, 42%; and grain supplementation, 1/19, 5%) (Figure 14B). The results demonstrate that dietary components significantly affected the potential of oocytes to reach the blastocyst stage of development.

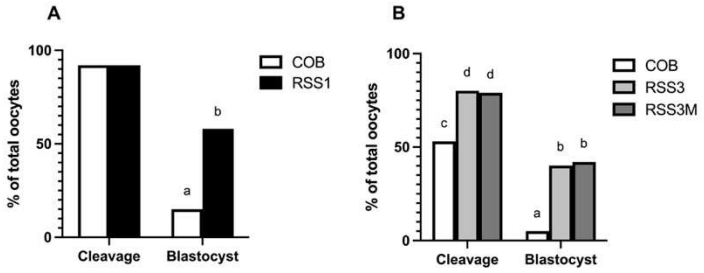

Figure 14: Embryonic development rates after intracytoplasmic sperm injection (ICSI). Cleavage rates (≥2 cell embryos per sperm-injected oocytes by 2 days after ICSI) and blastocysts rates (number of blastocysts per sperm-injected oocytes) for (A) oocytes from mares supplemented with grains (COB, n=13) or a reproductive support supplement with additional antioxidants (RSS1, n=12) and (B) oocytes from mares supplemented with grains (COB, n=19) or reproductive support supplements with n-3 PUFA (RSS3, n=15) or with the substitution of n-3 PUFA with n-6 PUFA (RSS3M, n=24). Bars with different superscripts differed (ab, P<0.05 or cd, P<0.1) for the same end point.

Discussion

The extent that diet supplements can affect female fertility is dependent on their potential to influence reproductive tissues directly or indirectly, with the oocyte being one of the most important and difficult of cells to impact. In the present study, we examined the potential for dietary supplements, designed to support health and reproductive function and fed for approximately two months, to affect oocyte metabolic function, lipid content, and developmental potential in older mares. We used the mare, a monogastric large animal with an easily manipulated reproductive tract, for our studies. Maternal aging has a marked effect on mare

reproductive efficiency, with a decline beginning in the early teen years (Ginther, 1992). Although many mares will cycle into their twenties, their fertility is poor and associated with a decline in oocyte developmental competence (Carnevale and Ginther, 1995). Considering the similarities between the mare and woman in follicular development (e.g., monovular, long follicular phase, similar follicle wave patterns, decades-long reproductive lifespans) and age-associated changes in reproduction, the mare represents an applicable model for reproductive aging in women (Carnevale, 2008; Carnevale *et al.*, 2020) and vice versa.

Assisted reproductive technologies are often used in the mare and woman to produce offspring from subfertile females. However, in both species, oocyte developmental potential can be low, especially in older oocyte donors (Navot *et al.*, 1991; Carnevale and Ginther, 1995), and assisted reproductive procedures are costly and not always practicable. Diet supplementation represents a feasible approach *in vivo* to improve female reproductive outcomes, especially if developmental ability of the oocyte that is destined to ovulate can be improved. Specific compounds have been studied as to their effect on reproductive parameters, although more information is available for the woman than the mare. However, our primary goal was to determine if and to what extent the follicle and oocyte could be impacted by dietary supplements and not to study individual components, as nutrient function can be codependent and synergistic. The base diet for the studies was hay of a quality consistent with that fed to mares under maintenance conditions and for reproductively active mares. Treatment groups were provided a nutritional foundation of vitamins, minerals, pre- and pro-biotics for digestive support, and proprietary reproductive support supplements with additional antioxidant and cell metabolic support nutrients. Some of these nutrients have been reported to improve stallion semen quality, including d-alpha-tocopheryl acetate, n-3 PUFAs, and L-carnitine (Deichsel *et al.*, 2008; Contri

et al., 2011; Schmid-Lausigk and Aurich, 2014; Ruiz *et al.*, 2021). The effects of these compounds on gamete quality in the mare have not been definitively assessed. Our study used multiple and novel endpoints to demonstrate that the dietary intervention resulted in both systemic and follicular effects, culminating in oocytes from older mares having significant changes in metabolic function, lipid composition, and developmental potential.

The follicle and associated cells support the oocyte and can provide an indication of oocyte quality. Elevated follicular concentrations of ROS can reflect the active metabolism of a healthy follicle (Zarezadeh *et al.*, 2019); however, excessive levels of ROS are unfavorable for oocyte quality and embryo development, as observed for obesity, diabetes, and aging in women (Jančar *et al.*, 2007; Karuputhula *et al.*, 2013; Gu *et al.*, 2015; Lai *et al.*, 2018). The primary difference that we observed when older mares were fed a reproductive support supplement with additional antioxidants instead of grain was a significant decrease in ROS production relative to aerobic metabolism in granulosa cells. Reduced ROS production by granulosa cells could indicate a healthier follicular environment for oocyte development and maturation, as oxidative stress has a major negative impact on female fertility and oocyte health (Devine *et al.*, 2012). In this study, we only examined aerobic metabolism, although the extent that aerobic versus anaerobic metabolism is used by equine granulosa cells is not known. In the pig, another species having oocytes with abundant lipids, granulosa cells generate energy mostly via anaerobic glycolysis (Kansaku *et al.*, 2017). Further studies are needed to determine the metabolic preferences of equine granulosa cells.

We used novel microsensors to examine aerobic and anaerobic metabolism in single oocytes and early embryos (Obeidat *et al.*, 2018, 2019). Oocytes primarily generate energy through mitochondrial aerobic metabolism, resulting in the consumption of oxygen (May-

Panloup *et al.*, 2007). Aerobic metabolism, under physiological conditions, is controlled by energy demand and can be measured as basal oxygen consumption rate (OCR). The maximal aerobic metabolic potential provides information as to how much aerobic energy can be produced; it can be measured after the addition of mitochondrial uncoupler agents (Brand and Nicholls, 2011). In the present study, oocyte basal aerobic metabolism was significantly higher for oocytes from mares fed the reproductive support supplement than those fed grain, with aerobic metabolism of oocytes from supplemented mares comparable to the same endpoint in oocytes from young mares in a previous study (Catandi *et al.*, 2021). In agreement with the previous study (Catandi *et al*., 2021), higher oocyte aerobic metabolic activity was associated with more oocytes capable of developing into blastocysts. Although basal aerobic metabolism was reduced in grain-fed mares, maximal aerobic metabolism was similar between the two groups. Consequently, oocytes from mares fed grain had a similar potential to produce energy through aerobic metabolism; however, they were using significantly less of their energy potential under basal conditions than oocytes from mares fed the reproductive support supplement. In intact cells, basal mitochondrial metabolism is limited by substrate availability and regulated by energy demand (Brand and Nicholls, 2011). Reduced basal oxygen consumption in oocytes from mares fed grain supplements may, therefore, be associated with altered energy sensing cellular mechanisms, such as AMP-activated protein kinase (AMPK). This enzyme senses energy status and regulates anabolic and catabolic pathways to equilibrate ATP production and substrate consumption inside the cell and to regulate progression of oocyte maturation (Abdulhasan *et al.*, 2017; Yang *et al.*, 2020). In oocytes, AMPK activity is altered by maternal metabolic dysfunctions, such as diabetes (Ratchford *et al.*, 2007), and can be influenced by diet, although precise mechanisms have not been elucidated (Gu *et al.*, 2015). Oocyte AMPK activity was not

assayed in this study, but we speculate that diet supplementation with grains could have a negative impact, as seen with high-fat diets in multiple other tissues (Lindholm *et al.*, 2013). In addition, ingredients in the reproductive support supplement could have had a positive impact on AMPK function in oocytes, as seen after stimulation of lipid metabolism or addition of L-carnitine to oocyte *in vitro* maturation (Downs *et al.*, 2009; Downs, 2015). Higher oocyte basal mitochondrial metabolism and reduced lipid abundance observed after diet supplementation with the reproductive support supplement are consistent with this postulation.

Basal anaerobic metabolism (based on ECAR) was similar for the two groups, indicating that the grain-fed mares' oocytes did not try to compensate for lower aerobic metabolism by increasing anaerobic energy production. It remains unclear whether oocytes have the ability to recognize limitations in mitochondrial energy production and compensate by increasing their reliance on anaerobic glycolysis, as observed in muscle cells during intense exercise and hypoxia (Bowtell *et al.*, 2014). Regardless, almost 90% of energy produced in equine oocytes is provided by aerobic pathways (Lewis *et al.*, 2020), thus anaerobic energy production would likely not be enough to compensate for mitochondrial dysfunction. Ultimately, this suggests that the oocytes from the mares provided the reproductive support supplement had overall higher energy production than those fed grains. In addition, oocytes from mares fed the diet supplement were capable of more stimulated anaerobic metabolism, suggesting better metabolic flexibility to produce energy. Overall, oocytes from the grain-fed mares performed more anaerobic glycolysis as a proportion of their total energy production when compared to oocytes from mares fed the support supplement. This metabolic adaptation could affect embryo development, as the oocytes are using more of their glucose and pyruvate reserves when they should be more dependent on β-oxidation for energy production (Lewis *et al.*, 2020).

Copy numbers of mtDNA were determined as an estimate of oocyte mitochondrial numbers, although the association between mtDNA copy numbers and oocyte metabolic potential is not well known. In the present study, mtDNA copy numbers were significantly higher in oocytes from mares fed grain than the reproductive support supplement, although basal aerobic metabolism was higher in the later. Oocytes are not able to activate mitophagy in response to mitochondrial damage (Boudoures *et al.*, 2017); therefore, mitochondrial dysfunction and metabolic stress can lead to an abnormal, compensatory increase in mtDNA copy numbers (DiMauro and Schon, 2003; Meldrum *et al.*, 2016). In a previous study from our group, oocytes obtained from young versus old mares had higher basal and maximal aerobic metabolism, although no difference was noted for mtDNA copy numbers, confirming that oocyte mtDNA content is not indicative of mitochondrial function or oocyte quality (Catandi *et al.*, 2021). Our results are consistent with findings in women. Mature oocytes from young women have fewer mtDNA copy numbers but greater mitochondrial membrane potential when compared to oocytes from older women (Pasquariello *et al.*, 2019), further supporting that mtDNA copy numbers and mitochondria activity are not positively related.

Fatty acids have been suggested to be the primary substrate for oxidative energy production in equine oocytes (Lewis *et al.*, 2020). The reproductive support supplement provided additional PUFAs and L-carnitine. Within mitochondria, L-carnitine is essential for fatty acid β-oxidation, acting as a co-factor in the rate-limiting step involving the transport of activated fatty acids into mitochondria (Dunning and Robker, 2012). Additional dietary lipids may serve as an energy reserve for the oocyte; however, supplementation of these fatty acids alone has been associated with more negative than positive effects on oocyte quality in other species (Zarezadeh *et al.*, 2019). Short-term L-carnitine supplementation to ewes does not affect follicular

development and ovulation, while L-carnitine combined with long-chain fatty acid supplementation improves the number and size of preovulatory follicles and ovulation rates (El-Shahat and Abo-El maaty, 2010). Thus, there seems to be a synergistic effect of L-carnitine and fatty acids on the ovine ovaries and developing follicles. As lipid content varies in oocytes from different species (Dunning *et al.*, 2014), the extent that fatty acids are used as an energy substrate for the oocyte and the impact of L-carnitine could vary.

In the first experiment, the reproductive support supplement had additional antioxidants to offset the potential effects of aging, included CoQ10, pterostilbene, and PQQ. Aging impairs the expression of enzymes involved in the natural production of CoQ10 in multiple tissues, including follicular cells (Ben-Meir *et al.*, 2015), and dietary supplementation with CoQ10 is associated with improved oocyte mitochondrial function and developmental potential for aged mice (Ben-Meir *et al.*, 2015). Pterostilbene is associated with lowering the effects of oxidative stress in aging, and murine oocyte quality and maturation rates improve when supplemented during *in vitro* maturation (Li *et al.*, 2018; Ullah *et al.*, 2018). PQQ is a natural antioxidant that has been associated with improved reproductive performance when supplemented to female mice (Steinberg *et al.*, 2003). In our study, a positive synergistic effect of the antioxidants with other components of the reproductive support supplement could have occurred. However, results from all experiments strongly support that the overall diet supplement was the primary factor affecting oocytes, regardless of the addition of antioxidants.

In a second experiment, lipid content was compared for oocytes from the same mares prior to and after approximately 8 weeks of feeding a reproductive support supplement mixed with a pelleted complete feed and grain mix (RSS2). Oocyte lipid composition was altered after supplementation, with a pronounced reduction in triglyceride abundance. Lipids may serve as the

main substrate for aerobic energy production during oocyte maturation, as glucose is mostly directed for anaerobic energy production (Lewis *et al.*, 2020). Systemic and follicular triglyceride concentrations were consistent with a previous study from our group using the same methodology and follicle category (Sessions-Bresnahan *et al.*, 2016), with TG concentrations higher in plasma when compared to follicular fluid, although this relationship was not consistent with other equine studies (Collins *et al.*, 1997; Satué *et al.*, 2019). Triglyceride concentrations in follicular fluid were reduced after diet supplementation, although a similar decline was not noted in systemic concentrations. However, the diet supplement caused a significant decline in systemic free fatty acids. Omega-3 PUFA supplementation has been associated with systemic hypolipidemic effects (Madsen *et al.*, 1999), but it did not affect fatty acid concentrations in the serum of pregnant and lactating mares (Hodge *et al.*, 2017). The concentrations of L-carnitine in the present study were consistent with previous reports in equine follicular fluid and plasma (Foster *et al.*, 1988; Zeyner and Harmeyer, 1999; Fernández-Hernández *et al.*, 2020). A short-term increase in systemic L-carnitine occurs after oral ingestion in horses; however, plasma concentrations are only increased for a few hours after ingestion (Zeyner and Harmeyer, 1999). We collected blood samples in the morning prior to consumption of supplements, potentially missing any transitory increase in systemic L-carnitine; however, follicular fluid samples were collected in the late morning or early afternoon after supplements were fed in the morning. Therefore, although the results demonstrate that dietary supplementation altered oocyte lipid content, further studies are needed to determine if the effect was primarily caused by follicular or systemic alterations.

Basal and maximal OCR from early embryos were consistently higher than values observed for oocytes, as noted in a previous study (Catandi *et al.*, 2021). During early embryonic

development, mitochondrial numbers do not change (Hendriks *et al.*, 2019), but the organelles go through morphological changes from the immature stage present in oocytes to more active stages (Bavister and Squirrell, 2000; Van Blerkom, 2011). However, no differences were observed between diet groups for day-2 embryo aerobic metabolism.

In the present experiments, oocyte developmental potential was determined by cleavage and blastocyst formation after ICSI. In our first experiment, cleavage and blastocyst rates were compared for mares that were fed grain or the diet supplement with additional antioxidants. Cleavage rates were not significantly different between groups, consistent with our previous finding when comparing cleavage rates after ICSI for young and old mares (Catandi *et al.*, 2021). However, blastocyst formation was significantly improved for mares fed a reproductive support supplement when compared to grain. Considering the mean age of the mares (18.5 years), the blastocyst rate for mares fed the reproductive support supplement (58%) was high when compared to rates obtained in a previous study using frozen-thawed sperm from the same stallion for ICSI (21% for old mares, ≥ 20 years, and 48% for young mares, ≤ 14 years) (Catandi *et al.*, 2021). In a final experiment, the developmental potential of oocytes from mares fed grain were compared to those of mares fed the reproductive support supplement or the same supplement after the substitution of most of the n-3 PUFAs with n-6 PUFAs. Regardless of the PUFA content, mares provided the diet supplement had similar cleavage and blastocyst formation rates. In agreement with these findings, embryonic development rates after *in vitro* fertilization of oocytes from dairy cows supplemented with n-3 PUFA for 3 months were higher when compared to cows fed a control diet, but not different from cows supplemented with n-6 PUFA (Zachut *et al.*, 2010). However, mares fed grain tended to have lower cleavage rate and had a significantly lower blastocyst formation rate, demonstrating that the final concentration of n-3 or

n-6 PUFA are not as crucial as other ingredients in the dietary supplementation for improving the developmental potential of oocytes from older mares.

In the current study, we did not try to identify the effect of one single nutrient on mare follicular metabolism and oocyte developmental potential. Instead, we compared supplementation with grain products, which remain popular feed ingredients in the equine industry and the Western human diet, to feed ingredients designed to support overall wellness and potentially support reproduction and mitochondrial function. Because we used a complex of nutrients, we cannot differentiate individual versus synergistic effects of supplement components on the associated differences in oocyte metabolic function and lipid composition. We are also unsure of the extent that the complex nutrients were beneficial versus even a limited amount of grain was detrimental to oocyte metabolism and developmental potential. However, we clearly observed that the diet components had a substantive effect on oocyte composition, metabolic function, and developmental potential. Consequently, we demonstrated that short-term diet additives can affect reproductive function at the cellular level in older mares, providing a feasible method and model to study the interaction of diet and reproduction in the female. Our results suggest that diet has the potential to alter reproductive outcomes in mares by ultimately having a direct effect on the ovarian follicle and oocyte.

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CHAPTER IV: FOLLICULAR METABOLIC ALTERATIONS ASSOCIATED WITH OBESITY IN MARES CAN BE MITIGATED BY DIETARY SUPPLEMENTATION[9]

Summary

Obesity is a growing concern in human and equine populations, predisposing to metabolic pathologies and reproductive disturbances. Cellular lipid accumulation and mitochondrial dysfunction play an important role in the pathologic consequences of obesity, which may be mitigated by dietary interventions targeting these processes. We hypothesized that obesity in the mare promotes follicular lipid accumulation and altered mitochondrial function of oocytes and granulosa cells, potentially contributing to impaired fertility in this population. We also predicted that these effects could be mitigated by dietary supplementation with a combination of nutrients, including L-carnitine and chromium, to improve follicular cell metabolism. Twenty mares were grouped as: Normal Weight (NW, n=6, BCS 5.7±0.3), Obese (OB, n=7, BCS 7.7±0.2), and Obese Diet Supplemented (OBD, n=7, BCS 7.7±0.2) fed specific feed regimens for ≥6 weeks before sampling. Granulosa cells, follicular fluid, and cumulus-oocyte complexes were collected from follicles ≥ 35 mm during estrus and after induction of maturation. Obesity promoted several mitochondrial metabolic disturbances in granulosa cells (excessive ROS production, greater mitochondrial damage, and altered substrate oxidation capacities), reduced L-carnitine availability in the follicle, promoted lipid accumulation in cumulus cells and oocytes, and increased basal oocyte metabolism. Diet supplementation

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mitigated most of the metabolic changes in obese mares, resulting in parameters similar to NW mares. In conclusion, obesity disturbs the equine ovarian follicle by promoting lipid accumulation and altering mitochondrial function. These effects may be at least partially mitigated by dietary supplementation with L-carnitine, chromium and other additives, thereby potentially improving fertility outcomes in obesity.

Keywords: Equine, oocyte, obesity, diet, metabolism, follicle, granulosa cell

Introduction

Obesity is a growing public health concern in the human population and is linked with metabolic disturbances and a predisposition to infertility (Silvestris *et al.*, 2018). Obese women undergoing clinical assisted reproduction technology (ART) procedures have reduced embryo development and lower pregnancy and live birth rates in comparison to normal-weight women (Shah *et al.*, 2011). Among the reproductive alterations promoted by obesity, changes in the follicular environment and oocyte seem to have a major impact (Andreas *et al.*, 2021). Granulosa cells line the ovarian follicle and play essential roles in metabolism and transport of nutrients from the systemic circulation to the follicular fluid, providing an appropriate local environment for the developing oocyte. Cumulus cells are a specialized type of granulosa cells that directly surround and nurture the oocyte through cellular communications (Richani *et al.*, 2021). Obese women tend to have more lipids in their ovarian follicles, and research models, both *in vivo* and *in vitro*, have demonstrated that prolonged exposure to elevated lipids lead to impaired oocyte developmental potential and could contribute to transgenerational transmission of metabolic diseases, as mitochondria from the oocyte give rise to all mitochondria in future offspring (Van Hoeck *et al.*, 2013; Turner and Robker, 2015; Andreas *et al.*, 2021).

Obesity is also observed in the equine population, with a prevalence of up to 50% in different regions of the USA (Pratt-Phillips *et al.*, 2010; Thatcher *et al.*, 2012; Ragno *et al.*, 2019; Harris *et al.*, 2020). Similar to humans, equine obesity increases the propensity for metabolic pathologies, particularly insulin dysregulation and metabolic syndrome (Johnson *et al.*, 2009; Holbrook *et al.*, 2012). Reproductive disturbances have been associated with obesity in the mare (Sessions *et al.*, 2004; Vick *et al.*, 2006), including lipid accumulation in the follicle and oocyte (Sessions-Bresnahan *et al.*, 2016). These can contribute to life-long effects on offspring health, as metabolic and inflammatory changes are observed in foals from obese mares (Morley and Murray, 2014; Robles *et al.*, 2018a). Although fetal exposure to obesogenic signals during gestation contributes to developmental programming of metabolic diseases, there is growing evidence that preconception alterations in the oocyte establish transgenerational transmission of obesity and insulin resistance (Turner and Robker, 2015). The mare is considered a strong animal model for investigation of the effects of maternal conditions on human reproduction due to several important similarities in reproductive physiology (Gastal *et al.*; Carnevale, 2017; Benammar *et al.*, 2021). Additionally, ART procedures similar to the ones clinically applied for humans are routinely performed in horses (Lazzari *et al.*, 2020), and the large equine preovulatory follicle allows abundant and relatively easy collection of follicular fluid and cells (Carnevale *et al.*, 2020).

We have previously used the mare to study the effects of maternal aging on follicular cells and oocyte metabolism (Catandi *et al.*, 2019a, 2020a, 2021) as well as to elucidate the potential of dietary supplements to mitigate these effects and improve oocyte metabolic function and quality (Catandi *et al.*, 2019b, 2020b, 2022). Microsensors were used to quantify aerobic and anaerobic metabolism from single oocytes, measured, respectively, as oxygen consumption rate

(OCR) and extracellular acidification rate (ECAR) (Obeidat *et al.*, 2018, 2019). Mare aging negatively affects oocyte mitochondrial function and oxidative capacity while also impairing anaerobic metabolism, collectively leading to a reduction in oocyte energy production and, consequently, in embryo development (Catandi *et al.*, 2021). In a follow up study, we demonstrated that supplementing older mares with a combination of nutrients designed to promoted gastrointestinal wellness and cellular metabolism improved oocyte metabolism, reduced oocyte lipid accumulation, and increased embryonic development after intracytoplasmic sperm injection (ICSI) (Catandi *et al.*, 2022).

Alterations in oocytes promoted by maternal obesity seem to be associated with lipid overload in the follicle; however, this ultimately leads to mitochondrial dysfunction and oxidative stress in granulosa cells and oocytes (Andreas *et al.*, 2021; Gonzalez *et al.*, 2022). Studies in humans and rodents demonstrate potential beneficial effects of certain dietary additives on cellular metabolism. Among studied feed ingredients, L-carnitine and chromium, alone or in combination, have been shown to improve insulin sensitivity, reduce circulating lipid concentrations, and attenuate oxidative stress in obese mice and women (Noland *et al.*, 2009; Muoio *et al.*, 2012; Vincent, 2017; Jamilian *et al.*, 2018, 2020). Obesity-induced mitochondrial dysfunction is associated with L-carnitine insufficiency, which can be restored by diet supplementation (Noland *et al.*, 2009; Seiler *et al.*, 2014). L-carnitine is a mitochondrial co-factor essential for oxidation of fatty acids and regulation of pyruvate oxidation. In obese mammals, excessive circulating lipids lead to excessive formation of long-chain acylcarnitine that efflux from mitochondria and accumulate in body fluids, limiting the availability of free L-carnitine inside mitochondria and, thus, restricting pyruvate oxidation (Seiler *et al.*, 2014). Although diet recommendations are available for women undergoing ART procedures, specific

recommendations especially for obese women, are still unclear, and similar recommendations are even more scarce for mares.

The objectives of the present study were to characterize changes in lipid profiles and metabolic function of cells in the ovarian follicle associated with obesity in mares, as well as to assess the potential of dietary supplementation to mitigate these changes. We hypothesized that obesity promotes lipid accumulation in the ovarian follicle and negatively affects mitochondrial metabolism in oocytes and granulosa cells. However, dietary supplementation with a combination of nutrients, including L-carnitine and chromium, would improve cell metabolic function in the follicles of obese mares, ultimately contributing to follicle and oocyte viability and, potentially, offspring health.

Results

Morphometric measurements of mares

To assure consistent group differences, mares were closely monitored and fed treatment diets for > 6 weeks prior to follicular sample collections. Mares in three treatment groups: Normal Weight (NW), Obese (OB) and Obese Diet Supplemented (OBD) were assessed at 2-week intervals for changes in body weight and for morphometric indicators of adiposity, including body condition score (BCS), percentage body fat, and cresty neck score (Supplementary Figure 2, Appendix III). Throughout the study, morphometric measurements were mostly consistent within groups. Although no differences were noted for body weight among groups at any time point (NW: 530.0 ± 6.5, OB: 587.3 ± 8.0, OBD: 576.6 ± 6.0, $P \geq 0.1$; Supplementary Figure 2A, Appendix III), BCS and percentage body fat were greater in OB and OBD than NW throughout the study (respectively: NW: 5.6 ± 0.1, OB: 7.6 ± 0.1, OBD: 7.4 ±

0.1, P≤0.009; 7.0 ± 0.3, OB: 13.3 ± 0.7, OBD: 12.2 ± 0.4, P≤0.04; Supplementary Figure 2B, C, Appendix III), and the same differences were noted in cresty neck score from week 6-12 (P≤0.04; Supplementary Figure 2D, Appendix III).

Granulosa cell mitochondrial function and enzyme gene and protein expression

Granulosa cells were collected by aspiration of preovulatory follicles after induction of follicular maturation for assessment of mitochondrial function using two Oxygraph-2k high-resolution respirometers (Oroboros Instruments, Innsbruck, Austria). Oxygen consumption rate (OCR) and hydrogen peroxide (ROS) release rate were measured in intact cells without the addition of exogenous substrate (basal conditions), and in digitonin-permeabilized cells enabling delivery of saturating concentrations of pyruvate (5 mM) or fatty acid (0.05 mM palmitoylcarnitine) in the presence of malate (1 mM) and adenosine diphosphate (ADP 2.5 mM) to determine maximal rates of OXPHOS-linked OCR and ROS release capacities supported by carbohydrate or fatty acid oxidation, respectively. While basal OCR did not differ among groups (P=0.4; Figure 15A), basal rates of ROS release were greater in OB than NW and OBD (P≤0.0005; Figure 15B, C). Maximal OXPHOS-linked OCR and ROS production capacities were similar among groups for both pyruvate and palmitoylcarnitine (P≥0.7; Figure 15D, E, G, H). However, when normalized to OCR ROS release during pyruvate-supported OXPHOS was greater in OB than OBD (P=0.04; Figure 15F), with a similar trend seen during palmitoylcarnitine-supported OXPHOS (P≥0.2; Figure 15I). Mitochondrial inner membrane damage tended to be greater in granulosa cells from OB than NW mares (P=0.06), indicated by a greater increase in OXPHOS-linked OCR following the addition of cytochrome *c,* with no significant difference noted between OBD and NW (P≥0.1; Figure 15J). Indices of OXPHOS coupling efficiency were calculated from OCR supported by pyruvate or palmitoylcarnitine in

the absence of ADP (LEAK) and presence of ADP (OXPHOS) as [1-(LEAK/OXPHOS)], which tended to reflect impaired coupling efficiency in OB compared to other groups, but this did not reach statistical significance (P≥0.1; Figure 15K, L). Finally, mitochondrial substrate preference, calculated as the OCR ratio of pyruvate:palmitoylcarnitine oxidation normalized to the maximal OXPHOS rate (OCR supported by substrates + succinate) of each sample, was greater in NW than OB (P=0.02; Figure 15M), reflecting a greater relative capacity of OB mitochondria to oxidize fatty acids over carbohydrates compared to NW.

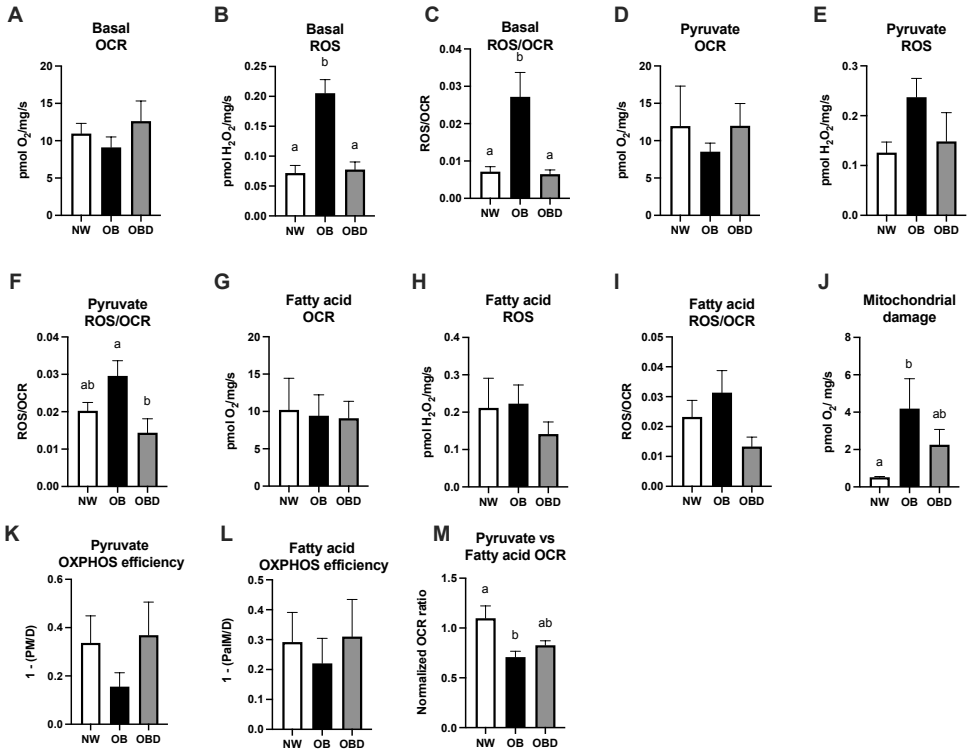

Figure 15: Effects of mare obesity and diet supplementation on granulosa cell mitochondrial function. Mitochondrial function of granulosa cells obtained from preovulatory follicles of normal-weight (NW, n=6), obese (OB, n=7) and obese diet supplemented (OBD, n=6) mares after ≥ 6 weeks of supplementation, expressed as the basal rates of oxygen consumption (OCR) (A), H_2O_2 (ROS) release (B), and ROS release as a proportion of OCR (ROS/OCR) (C) measured from intact cells under basal conditions; the carbohydrate oxidative phosphorylation (OXPHOS)-linked OCR (D), ROS release (E), and ROS/OCR (F) measured in permeabilized cells energized with 1 mM malate, 5 mM pyruvate, and 2.5 mM ADP; and the fatty acid OXPHOS-linked OCR (G), ROS release (H), and ROS/OCR (I) measured in permeabilized cells energized with 1 mM malate, 0.05 mM palmitoylcarnitine, and ADP.

Mitochondrial inner membrane damage assessed by the increase in maximal OXPHOS-linked OCR following the addition of cytochrome *c* (J). Indices of OXPHOS coupling efficiency [calculated as 1-(LEAK/OXPHOS OCR)] supported by pyruvate (K) and fatty acid (L) substrates. An index of carbohydrate versus fat oxidation capacity expressed as the pyruate:palmitoylcarnitine OCR ratio normalized to the maximal OXPHOS-linked rate as described in Methods (M). Graphs represent mean ± SEM. Different superscripts indicate differences among groups using one-way ANOVA with post-hoc Tukey's multiple comparison tests, or Kruskal-Wallis tests, followed by Dunn's multiple comparison tests ($P<0.05$).

To examine potential mechanisms for the observed effects of obesity and diet on granulosa cell mitochondrial function, protein expression of mitochondrial electron transport complexes and major cellular antioxidant enzyme were assayed in granulosa cells by western blot. While the relative protein abundance of each of the five electron transport complexes (I – V) in granulosa cells was not significantly different across groups ($P\geq0.4$; Figure 16B), the expression of ROS producing complexes (I and III) relative to non-ROS producing complexes (II, IV and V) was greater in granulosa cells from OB compared to NW and OBD ($P\leq0.03$; Figure 16C). Proportional protein abundance of complex I over complex II also tended to be greater in granulosa cells from OB than NW ($P=0.08$), while OBD was not different from the other groups ($P\geq0.2$; Figure 16D). Protein abundance of the ATP producing complex V (ATP synthase) in relation to abundance of all other complexes (I – IV) was not affected by mare group ($P\geq0.1$; Figure 16E), nor was the total protein expression of all electron transport system complexes ($P\geq0.8$; Figure 16F). Expression of the cytosolic superoxide dismutase isoform 1 was not different among groups ($P\geq0.9$; Figure 16H), while the mitochondrial superoxide dismutase isoform 2 was lower in granulosa cells from OBD than NW and OB ($P\leq0.04$; Figure 16I). Similarly, glutathione peroxidase 1 protein expression was lower in OBD compared to NW ($P=0.03$), but OB was not different from the other groups ($P\geq0.1$; Figure 16J).

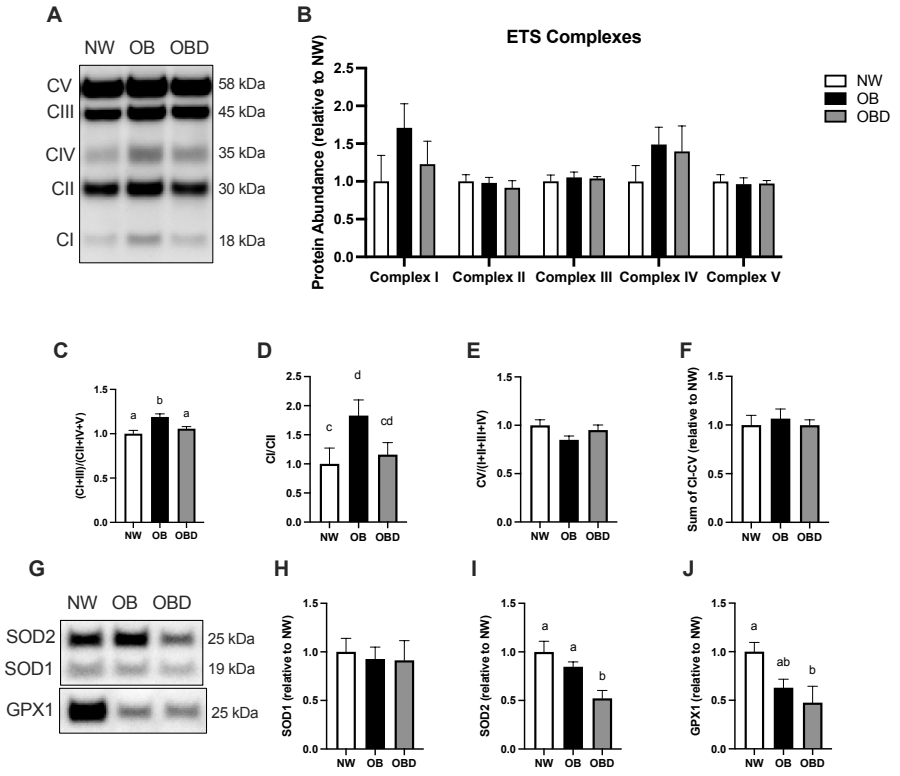

Figure 16: Effects of mare obesity and diet supplements on granulosa cell expression of mitochondrial proteins. Expression of mitochondrial complexes and antioxidant proteins in granulosa cells obtained from preovulatory follicles of normal weight (NW, n=5), obese (OB, n=5) and obese diet supplemented (OBD, n=5) mares after ≥ 6 weeks of supplementation; data are presented as fold changes relative to the control group (NW): (A) representative western blot of electron transport system complexes I – V, (B) expression of subunits from each of the electron transport system complexes, (C) ROS producing complexes (I and III) relative to other complexes, (D) complex I relative to complex II, (E) complex V (ATP synthase) relative other complexes (II, IV and V), and (F) the sum of all five complexes; (G) representative western blot of superoxide dismutase 2 (SOD2), superoxide dismutase 1 (SOD1), and glutathione peroxidase 1 (GPX1), (H) protein expression of SOD1, (I) SOD2, and (J) GPX1. Graphs represent mean ± SEM. Different superscripts indicate differences between groups at $P < 0.05$ (^{ab}) and $P < 0.1$ (^{cd}) using one-way ANOVA with post-hoc Tukey's multiple comparison tests.

Additional granulosa cells were assayed for expression of genes specific to pathways of interest. No differences were observed among groups for most of the genes assessed ($P \geq 0.1$; Supplementary Figure 3A – M, Appendix III). *CYP19A1* mRNA abundance was greater in granulosa cells from OB when compared to NW and OBD ($P \leq 0.004$; Supplementary Figure 3N, Appendix III).

Follicular fluid lipid and acylcarnitine abundance

The preovulatory follicle is filled with follicular fluid, providing a microenvironment for the developing oocyte. Follicular fluid is composed of plasma constituents that can cross the blood follicular barrier and follicular cell secretions. Follicular fluid samples were collected from the same preovulatory follicles as granulosa cells and cumulus-oocyte complexes and assessed for concentration of insulin, lipids and acylcarnitine. Concentration of insulin in follicular fluid was higher in both obese groups (OB and OBD) when compared to NW ($P \leq 0.02$; Figure 17A). Triglyceride and non-esterified fatty acid concentrations did not differ among the groups ($P \geq 0.4$; Figure 17B, C). Mare obesity did not significantly affect any short-, mid- or long-chain individual acylcarnitine species ($P \geq 0.1$; Table 2); however, diet supplementation to obese mares increased follicular concentrations of several acylcarnitines, mainly short-chain, in comparison to other groups ($P \leq 0.03$; Table 2). Abundance of total carnitines (sum of all acylcarnitine species and free L-carnitine), free L-carnitine, and acetylcarnitine tended to be less in OB than NW ($P = 0.09$) and was greater in OBD than both other groups ($P < 0.0001$; Figure 18A, B, C). Follicular concentration of the sum of all short-chain acylcarnitines was greater in OBD than NW and OB ($P \leq 0.0007$; Figure 18D). Total concentration of mid-chain acylcarnitines was greater in follicular fluid from OBD than OB ($P = 0.05$) and similar to NW ($P = 0.3$); OB and NW were not different ($P > 0.99$; Figure 18E). No group differences were noted for total long-chain acylcarnitine concentrations in follicular fluid ($P \geq 0.2$; Figure 18F). An elevated ratio between palmitoylcarnitine (C16) and propionylcarnitine (C3) is indicative of ineffective β-oxidation (Gervais *et al.*, 2015); the ratio was lower in OBD than OB ($P = 0.02$), with both similar to NW ($P \geq 0.2$; Figure 18G).

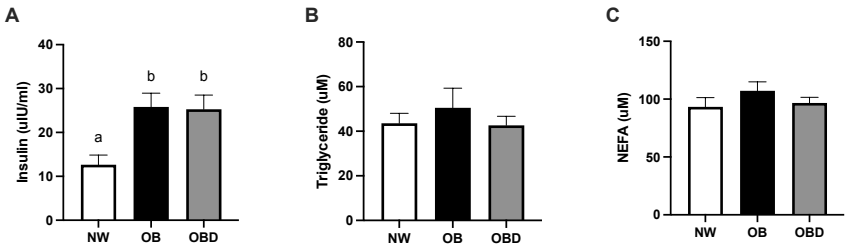

Figure 17: Effects of mare obesity and diet supplementation on follicular fluid insulin and lipid concentrations. Concentration of insulin and different lipid species in follicular fluid obtained from preovulatory follicles of normal-weight (NW, n=6), obese (OB, n=7) and obese diet supplemented (OBD, n=6) mares after ≥ 6 weeks of supplementation: (A) insulin , (B) triglycerides, and (C) non-esterified fatty acids. Graphs represent mean ± SEM. Different superscripts indicate difference (P<0.05) among groups using one-way ANOVA with post-hoc Tukey's multiple comparison tests.

Table 2: Concentration of individual acylcarnitine species (nM) in follicular fluid obtained from preovulatory follicles of normal-weight (NW, n=6), obese (OB, n=7) and obese diet supplemented (OBD, n=6) mares after ≥ 6 weeks of supplementation. Results are presented as mean ± SEM. Different superscripts within the same row indicate difference (^{ab}, P < 0.05) or tendency for difference (^{cd}, P < 0.1) between groups using one-way ANOVA with post-hoc Tukey's multiple comparison tests.

Short-chain acylcarnitines	NW	OB	OBD
Acetyl (C2)	25.48 ± 1.91^{ac}	20.37 ± 1.00^{ad}	47.41 ± 2.67^{b}
Propionyl (C3)	3.76 ± 0.71	2.35 ± 0.25	6.97 ± 0.75
Succinyl (C4-DC)	10.89 ± 1.96	11.39 ± 1.84	10.18 ± 1.78
Hydroxybutyryl (C4-OH)	1.93 ± 0.36	1.23 ± 0.30	2.48 ± 0.66
Butanoyl (C4)	3.55 ± 0.70^{ab}	2.44 ± 0.53^{a}	6.46 ± 1.00^{b}
Hydroxyisovaleryl (C5-OH)	0.18 ± 0.02	0.18 ± 0.03	0.27 ± 0.05
Isovaleryl (C5)	1.62 ± 0.15^{a}	1.12 ± 0.12^{a}	2.78 ± 0.11^{b}
Medium-chain acylcarnitines			
Adipyl (C6-DC)	0.27 ± 0.04	0.26 ± 0.05	0.35 ± 0.03
Hexanoyl (C6)	0.08 ± 0.01	0.06 ± 0.01	0.12 ± 0.02
Decanoyl (C10)	0.02 ± 0.001	0.02 ± 0.01	0.03 ± 0.003
Dodecanoyl (C12)	0.02 ± 0.002	0.02 ± 0.003	0.02 ± 0.002
Long-chain acylcarnitines			
Tetradecenoyl (C14:1)	0.05 ± 0.01	0.04 ± 0.01	0.07 ± 0.01
Tetradecanoyl (C14)	0.02 ± 0.02	0.02 ± 0.003	0.03 ± 0.003
Hexadecenoyl (C16:1)	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.02
Palmitoyl (C16)	0.17 ± 0.03	0.15 ± 0.02	0.16 ± 0.02
Linoleoyl (C18:2)	0.12 ± 0.03^{a}	0.25 ± 0.02^{ab}	0.37 ± 0.04^{b}
Octadecenoyl (C18:1)	0.43 ± 0.07	0.50 ± 0.07	0.50 ± 0.05

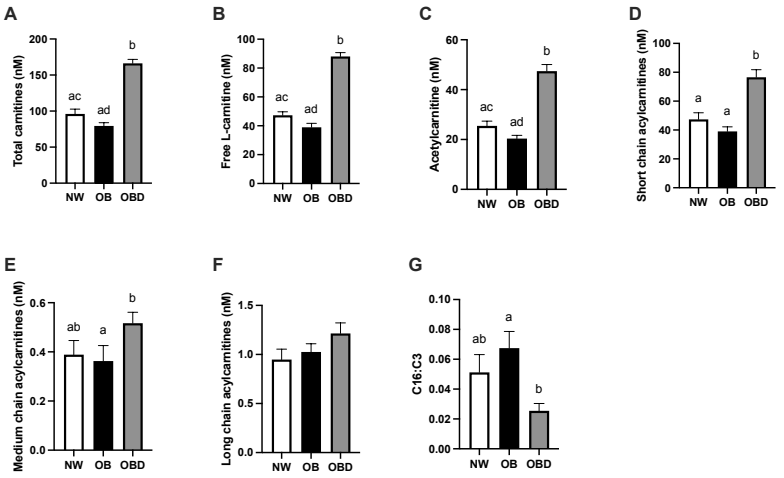

Figure 18: Effects of mare obesity and diet supplementation on follicular fluid concentration of acylcarnitines. Concentration of acylcarnitine species in follicular fluid obtained from preovulatory follicles of normal-weight (NW, n=6), obese (OB, n=7) and obese diet supplemented (OBD, n=6) mares after ≥ 6 weeks of supplementation: (A) total carnitine species (TC), (B) free L-carnitine (FC), (C) acetyl-carnitine (C2AC), (D) short-chain acylcarnitines (SCAC), (E) medium-chain acylcarnitines (MCAC), (F) long-chain acylcarnitines (LCAC), and (G) ratio of C16:C3 aceylcarnitines, indicative of complete β-oxidation rate. Graphs represent mean ± SEM. Different superscripts indicate difference (^{ab}, $P<0.05$) or tendency for difference (^{cd}, $P<0.1$) between groups using one-way ANOVA with post-hoc Tukey's multiple comparison tests.

Cumulus cell and oocyte lipid profiles

To determine the effects of mare obesity and diet supplementation on cumulus cell and oocyte lipid accumulation, cumulus-oocyte complexes were collected from preovulatory follicles, and oocytes and cumulus cells were separately assayed for lipid composition by liquid chromatography mass spectrometry. A total of 1,267 lipid species were identified in cumulus cells, from which 87 differed in abundance among the three groups (Supplementary Table 4, Appendix III). Of the 87 differing lipids, triglyceride was the most represented lipid class (44%). Lipid species were compared among groups as fold changes relatively to the control group (NW), and the mean fold change for each lipid class was compared among groups. We observed a general trend for lipid classes to be more abundant in cumulus cells from OB than NW and

OBD (Figure 19). The normalized abundance of triglycerides, acylcarnitines, phosphatidylcholines, phosphatidylethanolamines, lyso-phosphatidyletanolamines, sphingomyelins, phosphatidic acids and phosphatidylserines were significantly greater or tended to be greater in cumulus cells from OB than NW ($P \leq 0.08$); OBD was similar to NW and OB ($P \geq 0.2$; Figure 19A, D, E, G, H, J, M, O). Normalized abundance of diacylglycerols and lyso-phosphatidylcholines were significantly greater or tended to be greater in OB than NW and OBD ($P \leq 0.09$; Figure 19B, F). No significant differences were observed among groups for normalized abundance of non-esterified fatty acids, phosphatidylglycerols, cardiolipins, cholesteryl-esters, and phosphatidylinositols ($P \geq 0.2$; Figure 19C, I, K, L, N).

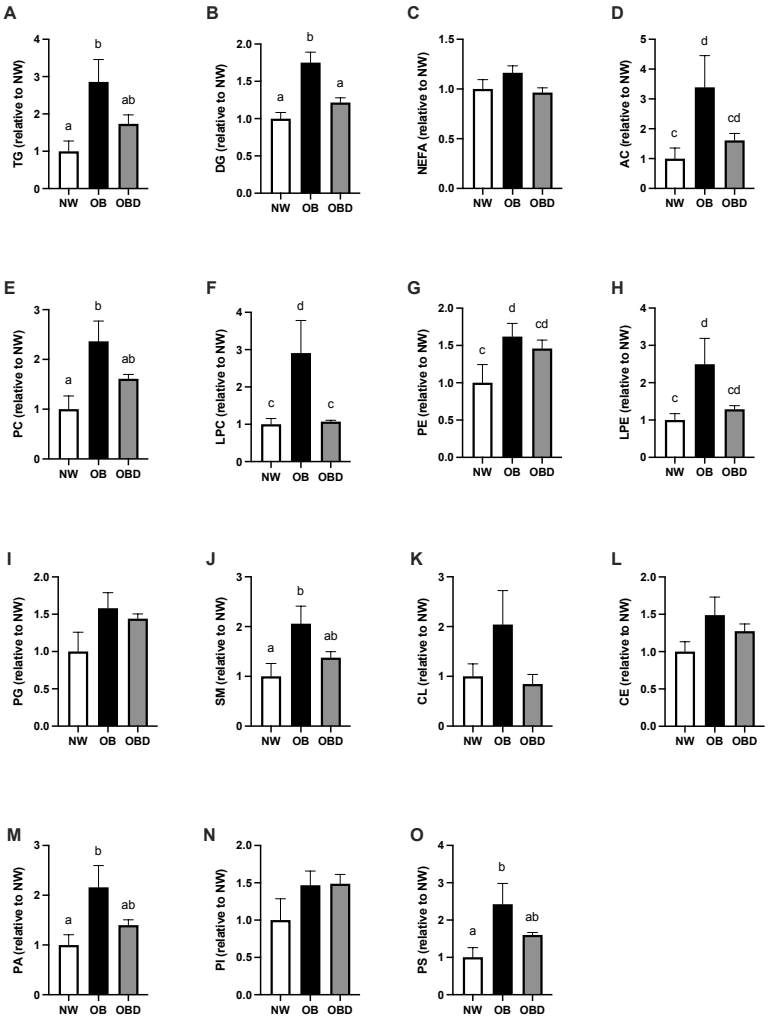

Figure 19: Effects of mare obesity and diet supplementation on cumulus cell lipid abundance. Relative abundance of different lipid species categories in cumulus cells obtained from preovulatory follicles of normal-weight (NW, n=6), obese (OB, n=7) and obese, diet-supplemented (OBD, n=6) mares after ≥ 6 weeks of supplementation, calculated as fold change relatively to NW: (A) triglycerides (TG), (B) diacylglycerols (DG), (C) non-esterified fatty acids (NEFA), (D) acylcarnitines (AC), (E) phosphatidylcholines (PC), (F) lyso-phosphatidylcholines (LPC), (G) phosphatidylethanolamines (PE), (H) lyso-phosphatidyletanolamines (LPE), (I) phosphatidylglycerols (PG), (J) sphingomyelins (SM), (K) cardiolipins (CL), (L) cholesteryl-esters (CE), (M) phosphatidic acids (PA), (N) phosphatidylinositols (PI), and (O) phosphatidylserines (PS). Graphs represent mean ± SEM. Different superscripts indicate difference (^{ab}, $P<0.05$) or tendency for difference (^{cd}, $P<0.1$) among groups using one-way ANOVA with post-hoc Tukey's multiple comparison tests.

In individual oocytes, a total of 335 lipid species were identified, from which 19 differed in abundance among groups (Supplementary Table 5, Appendix III). Similar to observations in cumulus cells, the most represented lipid class that differed among groups was triglyceride (42% of the individual lipid species that differed among groups). Fold change analyses for lipid classes were performed in oocytes and no differences were observed for the identified lipid classes ($P\geq0.2$; Figure 20A - L), although many of the lipids appeared to have a similar distribution for oocytes and cumulus cells.

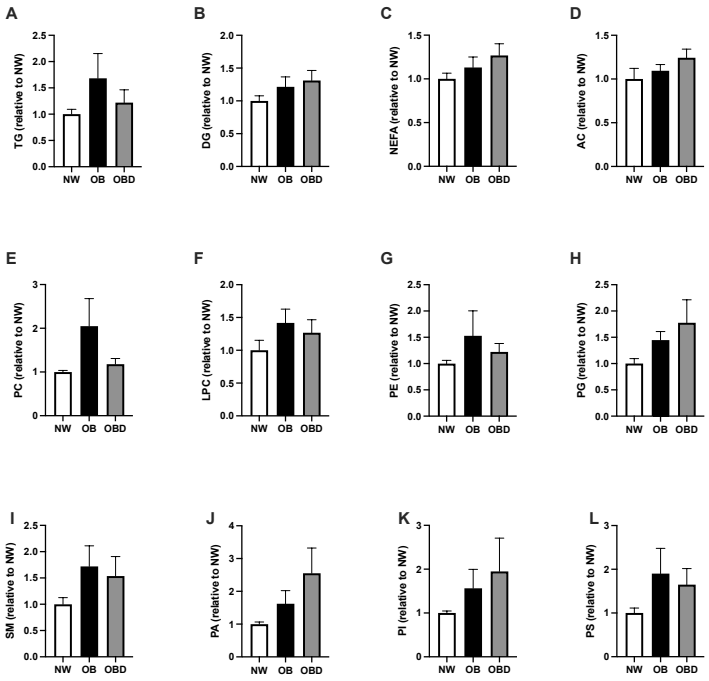

Figure 20: Effects of mare obesity and diet supplementation on oocyte lipid abundance. Relative abundance of different lipid species categories in oocytes obtained from preovulatory follicles of normal-weight (NW, n=6), obese (OB, n=7) and obese diet supplemented (OBD, n=6) mares after ≥ 6 weeks of supplementation, calculated as fold change relatively to NW: (A) triglycerides (TG), (B) diacylglycerols (DG), (C) non-esterified fatty acids (NEFA), (D) acylcarnitines (AC), (E) phosphatidylcholines (PC), (F) lyso-phosphatidylcholines (LPC), (G) phosphatidylethanolamines (PE), (H) phosphatidylglycerols (PG), (I) sphingomyelins (SM), (J) phosphatidic acids (PA), (K) phosphatidylinositols (PI), and (L) phosphatidylserines (PS). Graphs represent mean ± SEM. Differences were not significant (P≥0.2) among groups.

Oocyte metabolic function

Effects of maternal obesity on individual oocyte metabolism have not been determined in mares and other species. In the present study, mature oocytes were denuded of cumulus cells and assayed for basal aerobic and anaerobic metabolism, measured respectively as OCR and ECAR, using microsensors. Oocyte OCR was higher in OB than NW (P=0.04), and not different in OBD compared to NW and OB (P≥0.3; Figure 21A). Similar outcomes were noted for anaerobic metabolism, with higher ECAR in oocytes from OB relative to NW (P=0.05), and no difference between OBD and NW or OB (P≥0.2; Figure 21B).

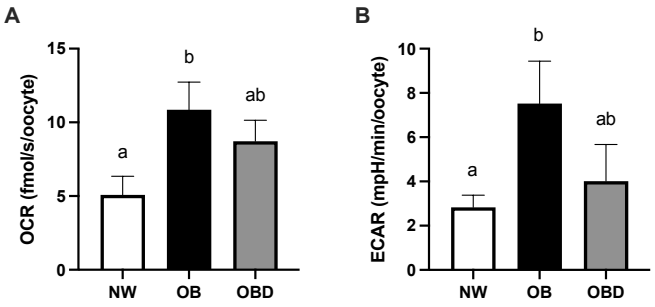

Figure 21: Effects of mare obesity and diet supplementation on oocyte metabolism. Basal metabolic function of oocytes obtained from preovulatory follicles of normal weight (NW, n=6), obese (OB, n=7) and obese diet supplemented (OBD, n=6) mares: (A) basal aerobic metabolism, measured as oxygen consumption rate (OCR) and (B) basal anaerobic metabolism, measured as extracellular acidification rate (ECAR). Graphs represent mean ± SEM. Different superscripts indicate difference among groups using one-way ANOVA with post-hoc Tukey's multiple comparison tests (P<0.05).

Discussion

Maternal obesity negatively affects fertility outcomes and may contribute to transgenerational transmission of metabolic diseases (Turner and Robker, 2015; Andreas *et al.*, 2021). Using several animal models, researchers have elucidated that obesity-induced alterations in the oocyte are mainly associated with lipid accumulation and the resultant mitochondrial dysfunction and oxidative stress (Igosheva *et al.*, 2010; Yang *et al.*, 2012; Van Hoeck *et al.*, 2013; Boots *et al.*, 2016; Sutton-McDowall *et al.*, 2016; Sessions-Bresnahan *et al.*, 2016). Improving oocyte mitochondrial function may help ameliorate oocyte and embryo quality from obese females and limit propagation of dysfunctional mitochondria to subsequent generations, as mitochondria from the oocyte give rise to all mitochondria in offspring (Turner and Robker, 2015). Diet interventions are a feasible *in vivo* approach to potentially improve female fertility. The present study was designed to examine the effects of mare obesity on metabolism of cells in the ovarian follicle and the potential of nutritional interventions to improve the follicular environment, specifically in terms of lipid accumulation and cellular metabolic function. The ovarian follicle creates a microenvironment around the oocyte, with the potential to alter the amount and type of substrates which are provided to the oocyte. The large size (approximately 45 mm in diameter prior to ovulation) of the equine follicle and the similar maturation timeline to the human follicle (Carnevale, 2008) provide the potential to explore the effects of excess adiposity on the ovarian follicle and potential treatment mechanism *in vivo*.

While increased follicular lipid concentrations are generally associated with obesity in women (Valckx *et al.*, 2014; Pantasri *et al.*, 2015; Gonzalez *et al.*, 2018), conflicting results have been reported (Valckx *et al.*, 2012; Mirabi *et al.*, 2017) and less is known about mares. In a previous study we reported a tendency for increased triglyceride concentration in follicular fluid

from obese when compared to normal-weight mares, as well as greater abundance of stearic and linoleic acids, although overall concentration of non-esterified fatty acids was not assessed (Sessions-Bresnahan *et al.*, 2016). Perhaps more sensitive assays, such as mass spectrometry utilized in our previous study, should be employed to identify group differences, which were not observed for triglycerides and non-esterified fatty acids in the present study. Studies that utilized similar methodology as performed in this study, reported no differences for triglyceride and non-esterified fatty acid concentrations in plasma from normal-weight and obese pregnant mares (Robles *et al.*, 2018a), or in mature horses after dietary induction of obesity (Ribeiro *et al.*, 2021).

Rodent and *in vitro* bovine models have demonstrated that exposure to high lipid concentrations during oocyte maturation led to lipid accumulation in cumulus cells (Wu *et al.*, 2010; Yang *et al.*, 2012; Lolicato *et al.*, 2015). Cumulus cell lipid accumulation is not always directly reflective of oocyte lipid accumulation, and there is evidence that the cumulus cell layer protects the oocyte from lipotoxic effects *in vitro* (Lolicato *et al.*, 2015). Associated with mare obesity, we observed an increased abundance of several lipid categories within cumulus cells, from which triglycerides were the most represented, as observed in the bovine *in vitro* model (Aardema *et al.*, 2013). Supportive data was reported by our group with increased gene expression of perilipin-2, which stimulates accumulation of lipid droplets, in cumulus cells from obese when compared to normal-weight mares (Sessions-Bresnahan *et al.*, 2016). Abundance of diacylglycerols, phosphatidylethanolamines and phosphatidylserines in cumulus cells of women undergoing ART procedures have been linked with prediction of negative pregnancy outcome (Montani *et al.*, 2012, 2013), and these lipids were found in greater abundance in cumulus cells from obese than normal-weight mares in the present study. However, significant differences

were not observed in oocytes. This potentially demonstrates a protective function of cumulus cells in preventing oocyte lipid accumulation, although the findings could also reflect a limited length of obesity and/or limited sample numbers. Additionally, advanced maternal age reduces abundance of transzonal projections in mice (El-Hayek *et al.*, 2018) and potentially in mares (Altermatt *et al.*, 2009), which could contribute to limiting transfer of lipids from cumulus cells to oocytes; in the present study, many of the mares were older (> 20 years). However, among lipid species that differed in oocytes between the groups, triglycerides were again the most represented, implying some degree of oocyte lipid accumulation promoted by obesity; this was reported previously in obese mares with increased abundance of some triglyceride species (Sessions-Bresnahan *et al.*, 2016). Equine oocytes are dark in appearance and are thought to be lipid laden, similar to pig oocytes (Dunning *et al.*, 2014). Triglyceride content in porcine oocytes decreases during maturation (Sturmey and Leese, 2003; Romek *et al.*, 2011), indicating that lipids are an important energy source for completion of maturation processes. Whether excessive lipid accumulation is detrimental for equine oocyte developmental potential remains to be determined.

L-carnitine and acylcarnitines directly participate in mitochondrial metabolism by serving as substrates to essential mitochondrial enzymes (Schooneman *et al.*, 2013). Systemically, L-carnitine insufficiency as promoted by obesity-related lipotoxicity seems to be the link between obesity and insulin resistance (Koves *et al.*, 2008; Schooneman *et al.*, 2013). In humans and rodent models, excessive lipids entering mitochondria form disproportionate quantities of long-chain acylcarnitines, which can cross membranes and accumulate in cells and circulation (Schooneman *et al.*, 2013). Whether maternal obesity promotes L-carnitine insufficiency in cells in the ovarian follicle remains unclear, but obese women have increased concentrations of long-

chain acylcarnitines in their follicles when compared to normal-weight women (Gervais *et al.*, 2015). This was not observed in the present study, in which follicular concentrations of individual or total long-chain acylcarnitines were not affected by mare obesity. Total mid- or short-chain acylcarnitines were also not affected. However, the tendency for lower total carnitines, free L-carnitine, and acetyl-carnitine in follicular fluid from OB when compared to NW suggests that L-carnitine insufficiency may limit carnitine acetyl-transferase activity, which could impair follicular cell mitochondrial metabolic flexibility, as suggested by the reduction in carbohydrate over fatty acid oxidation capacity observed in granulosa cells from obese when compared to normal-weight mares. Few studies have quantified acylcarnitine species in the follicular fluid of women (Várnagy *et al.*, 2013; Gervais *et al.*, 2015), and no reports are available for mares. Concentrations of all the reported acylcarnitine species and free L-carnitine seem to be much greater (200-500 times) in follicular fluid from women than mares, but the proportions of short-, mid- and long-chain species are similar. This can be, in part, a reflection of the difference in preovulatory follicular sizes, which is 2.1 times larger in mares than women (Ginther *et al.*, 2004). Interestingly C16:C3, which in humans is inversely related to complete oxidation of long-chain fatty acids (Gervais *et al.*, 2015), is much lower in mares than women. This may indicate a greater participation of lipid metabolism in the follicular environment from mares in comparison to women.

In accordance with a previous report by our group (Sessions-Bresnahan *et al.*, 2016), the insulin concentration in follicular fluid was greater for OB than NW. In women, follicular hyperinsulinemia promotes excessive androgen production and contributes to development of polycystic ovarian syndrome (Calcaterra *et al.*, 2021) and anovulation associated with increased follicular LH sensitivity and LH secretion (Gambineri, Alessandra *et al.*, 2019). Few equine

studies have quantified follicular insulin concentrations, but obesity and increased circulating insulin are associated with prolonged estrous cycle durations and anovulation in mares (Vick *et al.*, 2006). Although further investigations are needed to identify specific mechanisms, obesity-associated hyperinsulinemia seems to promote similar disruptions in ovarian activity for mares and women. The extent that follicular hyperinsulinemia directly affects the developing oocyte in the obese female is not known, as its effects are difficult to isolate from other obesity-induced alterations such as hyperlipidemia and oxidative stress. Nevertheless, bovine oocyte exposure to insulin during *in vitro* maturation impairs development and alters embryonic organization (Laskowski *et al.*, 2017).

Among the genes of interest assessed in granulosa cells, *CYP19A1* was the only gene in which mare obesity and diet supplementation altered transcription. Aromatase, the product of the *CYP19A1* gene, is a rate-limiting enzyme that catalyzes the conversion of androgens to estrogens during steroidogenesis. Generally, obesity, insulin resistance, and lipotoxicity have been associated with reduced aromatase gene and protein expression in women and mice (Belani *et al.*, 2018; Xu *et al.*, 2019; Hua *et al.*, 2020); but this has not yet been investigated in mares. In mares, granulosa cell expression of *CYP19A1* mRNA and the corresponding intrafollicular ratio of estradiol to progesterone start to decrease during the final stages of follicle development and, more intensely, just before ovulation, reflecting the luteinization of granulosa cells (Wischral *et al.*, 2022). Follicular aspirations for sample collection in the present study were performed after administration of a GnRH analog to induce follicular maturation; therefore, ovulation of the dominant follicle was expected to occur approximately 16 hours after the aspiration procedure. Accordingly, granulosa cells collected from such follicles are in the early stages of luteinization. We speculate that these changes may be delayed in granulosa cells from OB, which are

expressing elevated levels of *CYP19A1* mRNA in comparison to NW and OBD. Although not proven in women and mares, some have speculated that corpus luteum dysfunction associated with obesity (Kuokkanen *et al.*, 2016) may contribute to reduced fertility in obese females of such species (Silvestris *et al.*, 2018; D'Fonseca *et al.*, 2021).

Excessive accumulation of lipids in cells, promoted by obesity, leads to metabolic and mitochondrial overload, increased ROS production, and cellular oxidative stress (Hauck and Bernlohr, 2016). Accordingly, we observed several mitochondrial metabolic disturbances in granulosa cells from obese mares, namely excessive ROS production under basal and stimulated conditions, greater mitochondrial damage, and a trend for impaired OXPHOS efficiency especially when oxidizing pyruvate. Additionally, in obese mares, granulosa cell capacity to oxidize pyruvate compared to fatty acids was reduced, which may be associated with limited L-carnitine availability as discussed above. Interestingly, obesity also increased the expression of complexes I and III relative to the other complexes in the granulosa cell mitochondrial respiratory chain, possibly reflecting a stoichiometric shift in electron transport kinetics. The metabolic consequence of these shifts is unclear, but complexes I and III are the primary sites of ROS production in the mitochondrial respiratory chain (Li *et al.*, 2013), perhaps favoring the greater mitochondrial ROS release observed from OB granulosa cells.

Oocyte development in a lipotoxic environment is thought to be one of the main negative effects of maternal obesity on fertility, as it promotes direct impairment of oocyte quality because of lipid accumulation and metabolic and oxidative stress (Leroy *et al.*, 2022). In the present study, oocyte aerobic and anaerobic metabolism were significantly higher in OB than in NW mares, suggesting increased oocyte metabolic activity associated with obesity. While energy production is essential for oocyte function, excess oxygen consumption could result in an

increase in harmful byproducts, such as ROS, which has been shown to be detrimental to oocyte viability in obese mice (Igosheva *et al.*, 2010). To the best of our knowledge, no previous studies have described altered oocyte metabolism promoted by maternal obesity in mares, but a similar finding was recently reported in women. Oocytes from overweight women, assayed for aerobic metabolism through the same methodology utilized in this study, have higher OCR when compared to oocytes from normal-weight women (Cheng *et al.*, 2022a). In rodent models, diet-induced obesity increases oocyte mitochondrial membrane potential, mitochondrial damage, oxidative and endoplasmic reticulum stress (Igosheva *et al.*, 2010; Luzzo *et al.*, 2012; Wu *et al.*, 2015; Marei *et al.*, 2020). Although oocyte ROS formation was not directly assayed in the present study, increased metabolic rate observed in oocytes from obese mares could lead to oxidative stress and ultimately deleterious effects on oocyte quality.

Diet supplementation is a feasible method to influence oocyte quality *in vivo*. Recently, our group tested the potential of dietary supplementation to alter the follicular environment and improve oocyte quality in old mares (Catandi *et al.*, 2022). The dietary supplement formulation utilized in this study included the same equine wellness and digestion support components utilized in our previous study (trace minerals, vitamins, pre- and pro-biotics, omega-3 fatty acids, natural antioxidants, among others). It also included ingredients which specifically target obesity-induced metabolic disturbances, such as additional antioxidants (d-alpha-tocopherol and pterostilbene), L-carnitine and chromium, which are known to be metabolically beneficial to obese mice and humans (Noland *et al.*, 2009; Muoio *et al.*, 2012; Vincent, 2017; Jamilian *et al.*, 2018, 2020; Taherkhani *et al.*, 2021). Herein, we reported major positive effects of dietary supplements in lessening many of the negative effects of mare obesity in the ovarian follicle and oocyte. We also observed similar systemic effects, with improved insulin regulation and

mitochondrial function of muscle cells (unpublished data). While it is tempting to study individual nutrients or dietary components, the complex and synergistic mechanisms associated with metabolic health and cellular metabolic function guided our decisions to provide a complex blend of compounds to support cell metabolism.

Results from the current study demonstrate that dietary nutrient supplementation fed to obese mares promoted major metabolic improvements in granulosa cells. Excessive granulosa cell basal and OXPHOS-linked ROS production were prevented in obese mares receiving the dietary supplementation (OBD), which may be a direct effect of the supplemented antioxidants; however, mitochondrial oxidation efficiency was also improved. Expression of the primary sites of ROS production in the mitochondrial respiratory chain (complexes I and III) (Li *et al.*, 2013) was normalized in granulosa cells from OBD. Additionally, we observed evidence of reduced mitochondrial damage in OBD granulosa cells. Moreover, granulosa cell capacity to oxidize pyruvate, when compared to fatty acids was improved in OBD and comparable to NW. Potentially the diet supplement improved metabolic efficiency by increasing L-carnitine availability, which favors mitochondrial metabolic flexibility (Noland *et al.*, 2009). Lower expression of glutathione peroxidase 1 (GPX1) and the mitochondrial isoform of superoxide dismutase (SOD2) observed in OBD when compared to the other groups may also reflect lower mitochondrial ROS production when mares were fed the dietary nutrient supplementation, but elucidating the mechanistic links between these observations requires further investigation. Nevertheless, decreasing granulosa cell ROS release with dietary nutrient supplementation, as observed in the present study, may help preserve oocyte quality in maternal obesity. Feeding a multi-ingredient supplement, including antioxidants, vitamins and phytonutrients, was beneficial for the fertility of obese female mice (Nilsson *et al.*, 2023). Obese female mice fed a high fat diet

and the multi-ingredient supplement had reproductive outcomes comparable to normal weight females, while obese females fed the high fat diet and no supplements demonstrate higher ovarian inflammation, atretic follicles, and reduced number of litters (Nilsson *et al.*, 2023).

Concentrations of free L-carnitine and several acylcarnitine species were higher in follicular fluid from OBD than the other groups, indicating that L-carnitine supplemented in the diet reached the follicular environment. This could have improved the efficiency of mitochondrial metabolism in granulosa cells as suggested by our respirometry data and the lower ratio in OBD than OB of C16:C3 acylcarnitines, which in humans is indicative of more complete oxidation of long-chain fatty acids (Gervais *et al.*, 2015). By improving mitochondrial metabolic efficiency, L-carnitine could have contributed to reduction in ROS production by granulosa cells; however, L-carnitine also has direct antioxidant actions that can potentiate this effect (Surai, 2015). Taken together, these results support the conclusion that dietary supplements containing L-carnitine can at least partially mitigate impairments in mitochondrial metabolism of granulosa cells in the ovarian follicles of obese mares.

Chromium supplementation has been widely used in human medicine for management of obesity and diabetes, and has been associated with weight loss and improved glucose metabolism (Albarracin *et al.*, 2008), although its clinical relevance is still uncertain (Cefalu and Hu, 2004; Onakpoya *et al.*, 2013). In horses, chromium supplementation for 4 weeks improved insulin sensitivity of normal-weight animals fed carbohydrates daily (Spears *et al.*, 2020), but had no effect in obese horses supplemented for up to 16 weeks (Chameroy *et al.*, 2011). Chromium is thought to enhance insulin signaling pathways, participate in fat and carbohydrate metabolism, and manage oxidative stress; but specific mechanisms are still unknown (Onakpoya *et al.*, 2013; Morvaridzadeh *et al.*, 2022). Direct effects of dietary chromium on the ovarian follicle are still

largely undetermined, but systemic metabolic improvements seen in overweight and polycystic ovarian syndrome women may confer positive therapeutic fertility potential (Jamilian *et al.*, 2018, 2020).

Cumulus cell lipid profiles of obese mares were normalized with diet supplementation, which may contribute positively to oocyte quality. In women, there is a negative correlation between lipid content of granulosa and cumulus cells and pregnancy success after ART procedures (Raviv *et al.*, 2020). Although cumulus cell metabolism was not directly evaluated in this study, these cells are derived from and in direct contact to mural granulosa cells (Richani *et al.*, 2021). Metabolism of granulosa, cumulus cells, and oocytes are linked and regulated through bi-directional communication among the cell types (Su *et al.*, 2009). Thus, reduced and normalized lipids in cumulus cells from OBD may also be reflective of improved mitochondrial metabolism, as observed in the associated granulosa cells and oocytes.

Because mares in the OB group were fed grain additives to achieve obesity, we are unable to isolate potential negative effects of the grains from the effects of obesity on cellular metabolism. In terms of fetal development, both obesity and grain supplementation have negative effects that have been demonstrated in mares (Robles *et al.*, 2017, 2018a, b). Starch-rich diets adversely affect oocyte quality in cows (Leroy *et al.*, 2008; Rooke *et al.*, 2009) and may negatively affect fertility of women (Skoracka *et al.*, 2021). Nevertheless, grain additives are commonly included in the modern equine diet and predispose to obesity and metabolic diseases (Kaczmarek *et al.*, 2016). We are also unable to differentiate between individual and synergistic effects of the OBD dietary supplementation components. However, the beneficial effects of the supplements on cells of the ovarian follicles from obese mares demonstrated the potential of dietary interventions as an applicable therapy for obese women and mares facing reproductive

challenges. OBD mares were also fed grain additives, thus any potential negative effects of grain consumption on the follicular environment were overcome by the diet supplementation components. Correction of oocyte metabolic dysfunction promoted by maternal obesity may not only contribute to improved fertility outcomes but may also aid in limiting developmental programming of metabolic disturbances in offspring.

In conclusion, the data reported in this study elucidates how maternal obesity influences multiple aspects of the ovarian follicular environment in mares and how short-term diet interventions can aid in normalizing obesity-induced metabolic changes in the follicle. Further studies are needed to elucidate the translational aspects of our findings and potential effects of individual diet supplementation ingredients.

Methods

Experimental design and mare feeding regimens

Mare procedures were approved by Colorado State University's Institutional Animal Care and Use Committee. Nonlactating, light-horse mares (n=20, 11-22 years) were matched by age and divided into three groups considering body condition scores (BCS, 1-9) (Henneke *et al.*, 1983) upon start of the study. Mares in the Normal Weight group (NW, n=6, mean age 17.8 ± 1.8 years) had a BCS of 5-6 at the beginning of the study. Mares included in the overweight groups were not all initially considered obese and ranged in BCS from 6 to 8 when assigned to obese groups: Obese (OB, n=7, mean age 18.6 ± 1.5 years); and Obese Diet Supplemented (OBD, n=7, mean age 17.7 ± 1.4years). Groups were housed in adjacent dry lots. NW mares were fed grass/alfalfa mix hay at approximately 2% of body weight daily and 57 g daily of a commercial vitamin and mineral forage balancer (Purina® Free Balance® 12:12, Purina Animal

Nutrition, Gray Summit, MO, USA). OB and OBD mares were fed grass/alfalfa hay *ad libitum* and, twice daily, 28.5 g of the forage balancer, 0.75 kg of whole oats and 0.75 kg of cracked corn to increase their daily caloric intake and ensure achievement of obesity by sample collections. OBD mares also received twice daily commercially available supplements designed to support equine gastrointestinal health [Platinum Performance® GI (73.5 g), a combination of vitamins, trace minerals, amino acids, antioxidants, omega-3 fatty acids, prebiotics and probiotics], and a proprietary blend formulated to support cellular metabolism [20 g; including L-carnitine, chromium and antioxidants (Platinum Performance Inc., Buellton, CA, USA)]. Mares were monitored for body weight and multiple indicators of adiposity, including BCS (1-9) (Henneke *et al.*, 1983), percentage of body fat (calculated from the equation: 2.47 + 5.47 * tailhead fat in cm) (Kane RA *et al.*, 1987), and cresty neck score (0-5) (Carter *et al.*, 2009) every 2 weeks. Tailhead fat thickness was measured with a 10 MHz, linear-array transducer positioned approximately 7.6 cm cranial and 5 cm lateral from the tailhead; fat thickness in this area has the strongest correlation to BCS (Gentry *et al.*, 2004). Mares were provided group-specific feeding regimes for 6-10 weeks before follicular sample collections in August and September.

Sample collection from preovulatory follicles

Follicular maturation was induced during the follicular phase and when the dominant follicle was ≥ 35mm in diameter and uterine endometrial edema, consistent with estrogen, was observed by ultrasonography. Administration of a GnRH analog, histrelin in an aqueous base (0.5 mg, IM; Doc Lane, Lexington, KY, USA), was used to induce follicle and oocyte maturation. Follicular fluid, cumulus-oocyte complexes and granulosa cells were collected by transvaginal, ultrasound-guided follicular aspirations of dominant follicles at 20 ± 2 h after induction, as previously described (Carnevale, 2016). Follicular fluid samples were aliquoted and

stored at -80°C until assays. Granulosa cells were rinsed in flush solution (Vigro Complete Flush Solution, Vetoquinol, Fort Worth, TX, USA), vortexed and centrifuged at 750 x g for 5 min. The cell pellet was resuspended for 1 min in red blood cell lysis buffer at 37°C, before dilution in 3 mL holding medium (TCM 199 with Hank's salts and 10% fetal bovine serum), and was centrifuged at 750 x g for 5 min. The pellet was then resuspended in PBS, centrifuged at same previous settings, and either resuspended in 1 mL of flush solution and held at 4°C until high-resolution respirometry assays, or snap frozen and stored at -80°C for later protein and RNA isolation. For lipid composition analyses, recovered cumulus-oocytes complexes were held in hyaluronidase (80 U/mL) for a few minutes and separated by sequential pipetting with a stripper pipette. Oocytes and cumulus cells were then separately rinsed and fixed in 100 µL of 50% methanol solution, snap-frozen in liquid nitrogen, and stored at −80°C until mass spectrometry analyses. For metabolic function assays, cumulus-oocyte complexes were incubated in medium (TCM199 with Earle's salts with 10% fetal bovine serum and 25 µg/mL of gentamicin) at 38.2°C in 5% CO_2 and air for 22 ± 2 h. Matured oocytes were stripped of cumulus cells to confirm extrusion of the first polar body. For electrochemical measurements, oocytes were held in a MOPS-buffered medium (G-MOPS™, Vitrolife, Englewood, CO, USA) at 4°C until microsensor assays.

Granulosa cell high-resolution respirometry

Intact granulosa cells were resuspended in 250 µL of mitochondrial respiration medium (MiR05) containing (in mM) 0.5 EGTA, 3 $MgCl_2$ hexahydrate, 60 lactobionic acid, 20 taurine, 10 KH_2PO4, 20 HEPES, 110 sucrose, and 0.1% BSA, pH 7.1 with KOH, then added to a 2-mL chamber in an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) containing room air-saturated oxygen (~160 µM) in MiR05 maintained at 37°C while

stirring at 750 rpm. Results were normalized to the protein concentration of the granulosa cell sample pelleted at 10,000 x g for 10 minutes following each assay. Basal OCR and ROS release of intact cells was measured prior to permeabilization of cell membranes with digitonin (10 μg/mL) to provide mitochondrial access to cell-impermeable substrates. Mitochondrial oxidative phosphorylation (OXPHOS)-linked OCR was stimulated in permeabilized cells by the addition of 1 mM malate + 5 mM pyruvate or 1 mM malate + 0.05 mM palmitoylcarnitine in the presence of 2.5 mM adenosine diphosphate (ADP) to assess carbohydrate- and fatty acid-linked OXPHOS capacities, respectively. Maximal OXPHOS capacity was then measured following the addition of 10 mM succinate (fully saturating electron input through complex II), followed by the addition of 10 μM cytochrome *c* to assess mitochondrial membrane damage. Cytochrome *c* is not permeable to the outer mitochondrial membrane, and thus stimulates OCR (by donating electrons directly to cytochrome oxidase) in direct proportion to the extent of mitochondrial membrane damage present in the sample. The rate of reactive oxygen species (ROS) release from samples was measured simultaneously with OCR in the OXPHOS-linked state by monitoring the development of resorufin fluorescence produced by the interaction of hydrogen peroxide released by the sample with 10 μM Amplex Red in the presence of horseradish peroxidase (1 U/mL) as previously described (Li Puma *et al.*, 2020). ROS data are presented as rate of release per second, and as a proportion of concomitant OCR.

Granulosa cell gene expression

mRNA from frozen granulosa cell pellets were extracted using a TRIzol RNA isolation protocol. cDNA samples derived from 1000 ng of RNA and synthesized using Platinum™ PCR SuperMix. Gene expression was determined using quantitative polymerase chain reaction (qPCR) using SYBR Green (LightCycler 480 SYBR Green Master, Roche Diagnostics,

Indianapolis, IN) (see Appendix A.1 for the detailed method). Quantification of mRNA transcripts from each gene of interest was normalized to a housekeeping gene (*GAPDH*). The relative expression of each gene was calculated by the delta delta CT method (Pfaffl, 2001). Target genes were specific to pathways of interest. Primer details are listed in Supplementary Table 6 (Appendix III).

Granulosa cell protein isolation and expression

Frozen granulosa cell pellets were thawed and resuspended in M-PER™ Mammalian Protein Extraction Reagent lysis buffer containing Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail (100X). Homogenates were sonicated (Branson 250 Digital Sonifier Ultrasonic Cell Disruptor, Branson Ultrasonics Corporation), and centrifuged at 10,000 x g for 10 min at 4°C. Supernatants were transferred to new microcentrifuge tubes and stored at -80°C. Each sample was analyzed for total protein using Bicinchoninic acid (BCA) assay before immunoblotting.

40 μL samples containing 30 μg of granulosa cell proteins, Bolt™ Sample Reducing Agent, and 2x Laemmli Sample Buffer were added to 4-12% Bis-Tris polyacrylamide gels and electrophoresed for 1 h at 150 V. Protein was then transferred to polyvinylidene difluoride membranes, blocked in 5% non-fat milk for 1 h, then rocked overnight in 5% non-fat milk containing 1:1000 primary antibody at 4°C. Antibodies for mitochondrial electron transport system complexes (Total OXPHOS, MS604300, Abcam, Boston, MA), SOD1 and SOD2 (SOD-101 and SOD-111, Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada) and GPX1 (PA526323, Invitrogen, Thermo Fisher Scientific, Waltham, MA) were used . Membranes were washed for 5 min 3 times in Tris-buffered Saline + Tween (TBST; 20 mm Tris-base, 150 mm NaCl, pH 7.4) prior to addition of 5% non-fat milk containing 1:3000 secondary antibody.

Membranes were rocked for 1 h, washed in TBST, and incubated with chemiluminescence (SuperSignal™ West Dura Extended Duration Substrate, Thermo Fisher) at room temperature for 1 min prior to imaging. After imaging, membranes were stained for total protein using Amido Black and imaged. Target protein concentrations were quantified with densitometric analysis and standardized to Amido Black.

Determination of follicular fluid insulin, lipid and acylcarnitine concentrations

Triglyceride concentrations in follicular fluid samples were determined using a colorimetric assay kit (Cayman Chemical, Ann Harbor, MI, USA) according to kit instructions. The 96-well, non-treated microplate was read at 540-nm absorbance on a Synergy 2 microplate reader (Biotek, Agilent, Santa Clara, CA, USA). All samples were assayed on a single plate. The intra-assay coefficient of variation was 1.35%, and the minimal detectable concentration was 1 mg/dL. Concentrations of non-esterified free fatty acids and insulin in follicular fluid were determined by a reference laboratory (Clinical Pathology Laboratory, Cornell University Animal Health Diagnostic Center, Ithaca, NY). Analyses of follicular fluid acylcarnitine profiles were conducted by a reference laboratory (University of Colorado Anschutz Medical Campus School of Medicine Metabolomics Core, Aurora, CO, USA) as previously described (Reisz *et al.*, 2019).

Cumulus cell and oocyte lipidomics analyses by liquid chromatography coupled to mass spectrometry

Samples were lyophilized to remove water. 500 µL of cold 100% methanol spiked with 0.33 µg/mL Cholesterol 2,2,3,4,4,6-D6, 97-98% -d6 was added to each sample. Three blank samples were included in randomized order. Samples were briefly vortexed, sonicated in a cold bath for 5 min, then shaken at 4°C for 30 min, briefly sonicated, and centrifuged at 15,000 x g for 10 min at 4°C. 470 µL were recovered and dried under nitrogen. Oocyte extracts were

resuspended in 60 µL 2:1 methanol/toluene and cumulus cell extracts were resuspended in 20 µL 2:1 methanol/toluene. 20 µL from each oocyte sample were collected and pooled for an oocyte quality control (QC) sample. 5 µL were collected from each cumulus cell sample for a cumulus cell QC. The samples were then transferred to inserts for direct LCMS injection. One microliter of extract was injected onto a ACQUITY UPLC system (Waters, Milford, MA, USA) in randomized order with a pooled QC injection after every 6 samples, as previously described by this laboratory (Catandi *et al.*, 2022). XCMS (version 3.16.1) in R (version 4.1.2) was used for feature finding, retention time alignment, correspondence analysis, and peak filling (Smith *et al.*, 2006; Tautenhahn *et al.*, 2008). RAMClustR (version 1.2.2) in R (version 4.0.5) was used to normalize, filter, and group features into spectra (Broeckling *et al.*, 2014).

Oocyte metabolic function assays (OCR and ECAR)

Assays of oocyte metabolic function were performed using a microchamber with electrochemical-based oxygen and pH sensors. Fabrication of the electrochemical sensor chips and hardware in the device used in this study were previously reported (Obeidat *et al.*, 2019; Cheng *et al.*, 2022b). The microchamber was filled with 180 µL of MOPS-buffered medium (G-MOPS™) and placed inside an incubator at 38.5°C (Cheng *et al.*, 2022b). Approximately 15 min before the assay, each denuded oocyte was warmed to 38.5°C and pipetted onto the working electrode of the oxygen sensor, after oxygen and pH sensors had reached a steady baseline state. OCR and ECAR were measured, respectively, with amperometric and potentiometric sensors (Obeidat *et al.*, 2019).

Data analyses

GraphPad Prism 9.3.1 was used. Continuous data were analyzed for normality by Shapiro-Wilk tests. Repeated body weight, BCS, percentage body fat, and cresty neck score measures were analyzed within and among groups by two-way ANOVA with post-hoc Tukey's multiple comparison tests. One-way ANOVA with post-hoc Tukey's multiple comparison tests were used to compare normally distributed data sets, Kruskal-Wallis tests, followed by Dunn's multiple comparison tests were used for data that failed normality. Values of $P<0.05$ were considered significant, and $P\leq0.1$ was considered tending toward significance. Results are presented as mean ± SEM.

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CHAPTER V: CONCLUDING REMARKS AND FUTURE DIRECTIONS

Maternal conditions of mammalian females, such as advanced age and obesity, are known for negatively affecting reproductive outcomes by directly impacting the oocyte (Krisher, 2019; Moghadam *et al.*, 2021; Leroy *et al.*, 2022). In the mare, although there is evidence of advanced aging and obesity affecting fertility after natural (McDowell *et al.*, 1992) or assisted breeding (Carnevale *et al.*, 2010; Frank *et al.*, 2019; D'Fonseca *et al.*, 2021), less is known about specific effects on the ovarian follicle and oocyte. Findings reported in this dissertation characterized for the first time the direct effects of mare advanced aging and obesity on different aspects of oocyte and follicular cell metabolism. Moreover, additional findings here reported demonstrate the potential of short-term dietary supplementation as a method for improving follicular health and oocyte metabolism of old or obese mares.

Proper oocyte metabolism is crucial for successful early embryonic development (Chappel, 2013), but direct assessment of single oocyte metabolism has been technically challenging. In the experiments detailed in this dissertation, novel microsensors able to quantify aerobic and anaerobic metabolism of single oocytes and embryos were utilized (Obeidat *et al.*, 2018, 2019a, b; Cheng *et al.*, 2022), allowing for investigation of equine oocyte metabolism. The data obtained through this and complementary methods elucidate some of the cellular mechanisms that link maternal conditions to impaired oocyte quality. These findings contribute to understanding and fighting fertility issues of the mare, but also other mammalian species including women, since mares and women share many reproductive physiology and senescence similarities (Carnevale, 2008, 2017; Carnevale *et al.*, 2020; Benammar *et al.*, 2021).

In Project 1, we concluded that advanced age of the mare alters several aspects of oocyte metabolism, namely availability of energy substrates (free fatty acids), aerobic and anaerobic metabolic activity and capacity, ultimately hindering the ability of the oocyte to develop into an embryo. Then in Project 2, we observed that by improving oocyte metabolism through targeted dietary supplementation, we were able to also improve developmental potential of oocytes obtained from old mares to rates comparable to those for young mares in Project 1. Seen together, findings from these two projects suggest that oocyte metabolism might be the link between advanced age of the mare and impaired fertility. In Project 3, although oocyte developmental potential was not evaluated, we could observe that oocyte metabolism is also affected by mare obesity, yet in a different way than mare aging. Obesity in mares has been associated with reduced pregnancy rates after embryo transfer (D'Fonseca *et al.*, 2021), suggesting a link between altered oocyte metabolism and limited developmental competence. Dietary supplementation to obese mares with ingredients specifically targeting obesity-induced mitochondrial dysfunction was able to normalize, to some extent, oocyte and follicular cell metabolism, emphasizing the potential of this approach in mitigating fertility issues of obese mares.

A big limitation of the dietary supplementation experiments conducted in Projects 2 and 3 is the utilization of a multi-ingredient supplement formula, which prevents us from identifying specific feed additives and mechanisms of action that resulted in improved cell metabolism. During the consecutive experiments in Project 2, a few ingredients were removed or replaced from the RSS formulation, but there were still several components in the formula that individually or synergistically contributed to the observed benefits. To contribute to the

knowledge here obtained, future *in vivo* or *in vitro* studies should aim to investigate effects of individual additives included in the diet or culture media on oocyte quality.

In the past few years, similar to observed in the bovine embryo production industry, numbers of *in vitro* produced equine embryos have grown further compared to *in vivo* derived embryos (Viana, 2022). Interestingly, negative effects of mare aging on oocyte quality and developmental potential after ICSI seem to be less impactful when immature oocytes are collected from secondary follicles and matured *in vitro* (Cuervo-Arango *et al.*, 2019). Nevertheless, when comparing *in vivo* to *in vitro* matured equine cumulus-oocyte complexes, major changes have been described on metabolomic and transcriptomic profiles (Walter *et al.*, 2019; De La Fuente *et al.*, 2022), and overall blastocyst rates after ICSI are better when using *in vivo* matured cumulus-oocyte complexes. These observations emphasize the extensive adjustments that might happen in the oocyte when placed in an artificial environment during the crucial time of maturation, which may lead to changes in the embryo, fetus and offspring. The use of microsensors and other tools utilized in the studies described in this dissertation open doors for more detailed investigation on the potential effects of *in vitro* culture composition on oocyte metabolism. This could then be compared to the ideal *in vivo* maturation and correlated to oocyte developmental potential.

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APPENDIX I

Supplemental material Chapter II: Equine maternal aging affects oocyte lipid content, metabolic function and developmental potential.

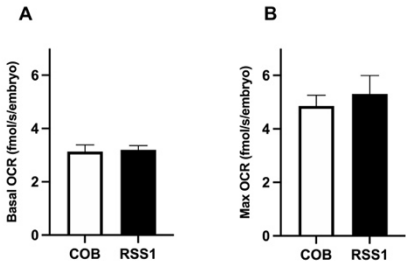
Supplementary Table 1: Relative abundance of triglycerides (TG) in MII oocytes and cumulus cells (CC). Single samples were analyzed from Young (n=8 mares) and Old (n=10 mares). Results are presented as mean ± SEM.

Triglyceride	Sample	Young		Old		P value
		Normalized abundance	Percentage of total TG	Normalized abundance	Percentage of total TG	
TG(50:1)	Oocyte	$4.90 \times 10^4 \pm 0.67$	12.99%	$7.04 \times 10^4 \pm 0.82$	15.37%	0.07
	CC	$3.36 \times 10^4 \pm 0.29$	4.48%	$3.85 \times 10^4 \pm 0.53$	3.50%	0.47
TG(54:5)	Oocyte	$3.41 \times 10^4 \pm 0.49$	9.04%	$3.85 \times 10^4 \pm 0.72$	8.42%	0.64
	CC	$1.18 \times 10^5 \pm 0.37$	15.64%	$1.65 \times 10^5 \pm 0.39$	15.02%	0.40
TG(56:7)	Oocyte	$1.24 \times 10^4 \pm 0.18$	3.29%	$1.17 \times 10^4 \pm 0.14$	2.56%	0.77
	CC	$5.70 \times 10^4 \pm 1.29$	7.58%	$9.22 \times 10^4 \pm 1.30$	8.39%	0.08
TG(54:6)	Oocyte	$1.45 \times 10^4 \pm 0.21$	3.85%	$1.49 \times 10^4 \pm 0.13$	3.25%	0.89
	CC	$3.69 \times 10^4 \pm 1.23$	4.90%	$5.91 \times 10^4 \pm 0.88$	5.38%	0.15
TG(55:2)	Oocyte	$5.28 \times 10^4 \pm 0.57$	14.00%	$7.09 \times 10^4 \pm 1.89$	15.48%	0.38
	CC	$1.93 \times 10^5 \pm 0.48$	25.62%	$2.98 \times 10^5 \pm 0.69$	27.08%	0.25
TG(51:2)	Oocyte	$2.51 \times 10^4 \pm 0.82$	6.66%	$2.49 \times 10^4 \pm 0.33$	5.43%	0.98
	CC	$9.98 \times 10^3 \pm 1.13$	1.33%	$1.16 \times 10^4 \pm 0.27$	1.06%	0.58
TG(58:6)	Oocyte	$5.55 \times 10^3 \pm 0.85$	1.47%	$5.75 \times 10^3 \pm 0.73$	1.26%	0.86
	CC	$1.45 \times 10^4 \pm 0.38$	1.93%	$2.27 \times 10^4 \pm 0.33$	2.06%	0.12
TG(58:8)	Oocyte	$2.30 \times 10^4 \pm 0.39$	6.09%	$2.46 \times 10^4 \pm 0.31$	5.37%	0.74
	CC	$2.94 \times 10^4 \pm 0.52$	3.91%	$4.43 \times 10^4 \pm 0.60$	4.02%	0.09
TG(58:9)	Oocyte	$3.19 \times 10^3 \pm 0.34$	0.84%	$3.55 \times 10^3 \pm 0.53$	0.78%	0.59
	CC	$2.42 \times 10^4 \pm 0.60$	3.21%	$3.95 \times 10^4 \pm 0.45$	3.59%	0.05
TG(49:2)	Oocyte	$1.44 \times 10^4 \pm 0.28$	3.82%	$2.02 \times 10^4 \pm 0.39$	4.40%	0.27
	CC	$6.66 \times 10^3 \pm 0.87$	0.89%	$8.13x\ 10^3 \pm 2.86$	0.74%	0.63
TG(54:7)	Oocyte	$8.52 \times 10^3 \pm 1.55$	2.26%	$9.51 \times 10^3 \pm 1.01$	2.08%	0.59
	CC	$1.35 \times 10^4 \pm 0.37$	1.79%	$2.16 \times 10^4 \pm 0.45$	1.97%	0.20
TG(52:5)	Oocyte	$1.54 \times 10^4 \pm 0.19$	4.08%	$1.60 \times 10^4 \pm 0.17$	3.49%	0.83
	CC	$1.98 \times 10^4 \pm 0.69$	2.63%	$2.87 \times 10^4 \pm 0.66$	2.61%	0.36
TG(56:8)	Oocyte	$5.92 \times 10^3 \pm 0.88$	1.57%	$5.57 \times 10^3 \pm 0.65$	1.22%	0.75
	CC	$2.12 \times 10^4 \pm 0.65$	2.81%	$3.74 \times 10^4 \pm 0.69$	3.40%	0.11
TG(58:5)	Oocyte	$4.21 \times 10^3 \pm 0.40$	1.12%	$5.06 \times 10^3 \pm 0.57$	1.10%	0.27
	CC	$5.80 \times 10^3 \pm 1.25$	0.77%	$7.99 \times 10^3 \pm 1.31$	0.73%	0.25
TG(48:1)	Oocyte	$2.66 \times 10^4 \pm 0.30$	7.04%	$4.07 \times 10^4 \pm 0.67$	8.90%	0.08
	CC	$1.57 \times 10^4 \pm 0.19$	2.09%	$2.04 \times 10^4 \pm 0.52$	1.85%	0.41
TG(56:5)	Oocyte	$1.38 \times 10^4 \pm 0.17$	3.67%	$1.46 \times 10^4 \pm 0.17$	3.18%	0.77
	CC	$4.53 \times 10^4 \pm 0.73$	6.02%	$6.59 \times 10^4 \pm 0.62$	6.00%	0.04
TG(54:8)	Oocyte	$2.71 \times 10^3 \pm 0.40$	0.72%	$3.43 \times 10^3 \pm 0.50$	0.75%	0.30
	CC	$2.73 \times 10^3 \pm 0.76$	0.36%	$4.59 \times 10^3 \pm 1.14$	0.42%	0.22
TG(56:4)	Oocyte	$5.84 \times 10^3 \pm 0.45$	1.55%	$7.22 \times 10^3 \pm 0.74$	1.58%	0.16
	CC	$1.53 \times 10^4 \pm 0.34$	2.04%	$2.30 \times 10^4 \pm 0.33$	2.10%	0.12

TG(54:4)	Oocyte	$4.81 \times 10^4 \pm 0.54$	12.76%	$5.44 \times 10^4 \pm 0.85$	11.87%	0.57
	CC	$8.04 \times 10^4 \pm 1.19$	10.69%	$9.58 \times 10^4 \pm 1.17$	8.71%	0.37
TG(49:1)	Oocyte	$9.63 \times 10^3 \pm 1.66$	2.55%	$1.42 \times 10^4 \pm 0.28$	3.09%	0.21
	CC	$5.74 \times 10^3 \pm 0.66$	0.76%	$8.39 \times 10^3 \pm 3.21$	0.81%	0.35
TG(62:14)	Oocyte	$2.37 \times 10^3 \pm 0.36$	0.63%	$1.97 \times 10^3 \pm 0.19$	0.43%	0.31
	CC	$4.08 \times 10^3 \pm 1.04$	0.54%	$6.18 \times 10^3 \pm 0.74$	0.56%	0.11

APPENDIX II

Supplemental material Chapter III: Oocyte metabolic function, lipid composition, and developmental potential are altered by diet in older mares.



Supplementary Figure 1: Aerobic metabolism, based on oxygen consumption rates (OCR), in embryos 2 days after intracytoplasmic sperm injection of oocytes from older mares supplemented with grain and corn oil (COB) or complex nutrients to support health and reproductive function (RSS1). (A) Basal OCR (COB, n=11; RSS1, n=12) and (B) maximal OCR (COB, n=6; RSS1, n=6). Barcharts present means ± SEMs.

Supplementary Table 2: Abundance of oocyte lipids that were affected by mare diet supplementation with RSS2. Single oocytes were analyzed from mares Pre and Post approximately two months of supplementation. Results are presented as mean ± SEM.

Lipid class	Lipid species	Pre	Post	P value
Triacylglycerols	TG(52:2)	$5.40 \times 10^6 \pm 0.42$	$2.71 \times 10^6 \pm 0.63$	0.049
	TG(52:3)	$4.27 \times 10^6 \pm 0.32$	$2.07 \times 10^6 \pm 0.49$	0.041
	TG(52:4)	$4.06 \times 10^6 \pm 0.35$	$2.26 \times 10^6 \pm 0.46$	0.047
	TG(50:2)	$3.52 \times 10^6 \pm 0.32$	$1.66 \times 10^6 \pm 0.35$	0.042
	TG(50:1)	$2.85 \times 10^6 \pm 0.34$	$1.36 \times 10^6 \pm 0.23$	0.049
	TG(54:3)	$2.34 \times 10^6 \pm 0.26$	$1.11 \times 10^6 \pm 0.27$	0.041
	TG(52:3)	$2.20 \times 10^6 \pm 0.16$	$1.11 \times 10^6 \pm 0.20$	0.039
	TG(52:2)	$2.02 \times 10^6 \pm 0.16$	$0.92 \times 10^6 \pm 0.19$	0.039
	TG(52:1)	$1.71 \times 10^6 \pm 0.16$	$0.79 \times 10^6 \pm 0.18$	0.039
	TG(50:3)	$1.65 \times 10^6 \pm 0.14$	$0.86 \times 10^6 \pm 0.18$	0.049
	TG(54:2)	$1.34 \times 10^6 \pm 0.17$	$0.60 \times 10^6 \pm 0.15$	0.041
	TG(56:7)	$0.91 \times 10^6 \pm 0.12$	$0.38 \times 10^6 \pm 0.08$	0.041
	TG(58:7)	$0.83 \times 10^6 \pm 0.09$	$0.41 \times 10^6 \pm 0.08$	0.039
	TG(50:4)	$0.81 \times 10^6 \pm 0.07$	$0.45 \times 10^6 \pm 0.10$	0.05
	TG(56:6)	$0.63 \times 10^6 \pm 0.09$	$0.24 \times 10^6 \pm 0.05$	0.039
	TG(54:7)	$0.60 \times 10^6 \pm 0.05$	$0.33 \times 10^6 \pm 0.07$	0.049
	TG(54:5)	$0.53 \times 10^6 \pm 0.04$	$0.26 \times 10^6 \pm 0.05$	0.039
	TG(51:2)	$0.47 \times 10^6 \pm 0.03$	$0.22 \times 10^6 \pm 0.04$	0.036
	TG(58:8)	$0.39 \times 10^6 \pm 0.06$	$0.16 \times 10^6 \pm 0.04$	0.047
	TG(49:2)	$0.37 \times 10^6 \pm 0.03$	$0.22 \times 10^6 \pm 0.04$	0.041

	TG(49:1)	$0.34 \times 10^6 \pm 0.02$	$0.18 \times 10^6 \pm 0.03$	0.044
	TG(51:3)	$0.33 \times 10^6 \pm 0.03$	$0.16 \times 10^6 \pm 0.03$	0.039
	TG(51:1)	$0.24 \times 10^6 \pm 0.02$	$0.12 \times 10^6 \pm 0.03$	0.041
	TG(53:2)	$0.23 \times 10^6 \pm 0.02$	$0.11 \times 10^6 \pm 0.02$	0.039
	TG(60:9)	$0.22 \times 10^6 \pm 0.02$	$0.11 \times 10^6 \pm 0.02$	0.036
	TG(52:2)	$0.21 \times 10^6 \pm 0.03$	$0.06 \times 10^6 \pm 0.02$	0.042
	TG(58:10)	$0.21 \times 10^6 \pm 0.02$	$0.11 \times 10^6 \pm 0.02$	0.041
	TG(52:2)	$0.18 \times 10^6 \pm 0.02$	$0.07 \times 10^6 \pm 0.02$	0.041
	TG(56:2)	$0.15 \times 10^6 \pm 0.01$	$0.07 \times 10^6 \pm 0.01$	0.048
	TG(51:5)	$0.14 \times 10^6 \pm 0.01$	$0.07 \times 10^6 \pm 0.01$	0.041
	TG(49:3)	$0.13 \times 10^6 \pm 0.01$	$0.07 \times 10^6 \pm 0.01$	0.048
	TG(60:2)	$0.12 \times 10^6 \pm 0.01$	$0.06 \times 10^6 \pm 0.02$	0.05
	TG(55:7)	$0.11 \times 10^6 \pm 0.01$	$0.06 \times 10^6 \pm 0.01$	0.041
	TG(52:1)	$0.10 \times 10^6 \pm 0.01$	$0.03 \times 10^6 \pm 0.008$	0.041
	TG(48:1)	$0.05 \times 10^6 \pm 0.01$	$0.02 \times 10^6 \pm 0.004$	0.05
	TG(60:6)	$0.02 \times 10^6 \pm 0.003$	$0.009 \times 10^6 \pm 0.002$	0.041
	TG(73:0)	$0.016 \times 10^6 \pm 0.001$	$0.009 \times 10^6 \pm 0.001$	0.041
	TG(56:3)	$0.013 \times 10^6 \pm 0.001$	$0.007 \times 10^6 \pm 0.0008$	0.039
	TG(53:1)	$0.007 \times 10^6 \pm 0.001$	$0.003 \times 10^6 \pm 0.0006$	0.048
Diacylglycerols	DG(36:2)	$3.55 \times 10^5 \pm 0.46$	$1.34 \times 10^5 \pm 0.30$	0.039
	DG(32:1)	$2.67 \times 10^5 \pm 0.39$	$1.17 \times 10^5 \pm 0.35$	0.039
	DG(36:2)	$2.23 \times 10^5 \pm 0.29$	$0.80 \times 10^5 \pm 0.15$	0.039
	DG(44:9)	$1.95 \times 10^5 \pm 0.26$	$0.71 \times 10^5 \pm 0.10$	0.039
	DG(40:8)	$1.74 \times 10^5 \pm 0.36$	$0.46 \times 10^5 \pm 0.11$	0.039
	DG(36:4)	$1.51 \times 10^5 \pm 0.23$	$0.64 \times 10^5 \pm 0.15$	0.05
	DG(34:2)	$1.32 \times 10^5 \pm 0.17$	$0.49 \times 10^5 \pm 0.10$	0.036
	DG(36:2)	$1.07 \times 10^5 \pm 0.08$	$0.57 \times 10^5 \pm 0.10$	0.039
	DG(40:8)	$0.84 \times 10^5 \pm 0.12$	$0.27 \times 10^5 \pm 0.04$	0.039
	DG(40:7)	$0.80 \times 10^5 \pm 0.17$	$0.38 \times 10^5 \pm 0.07$	0.05
	DG(38:4)	$0.74 \times 10^5 \pm 0.14$	$0.35 \times 10^5 \pm 0.08$	0.039
	DG(40:0)	$0.73 \times 10^5 \pm 0.08$	$0.35 \times 10^5 \pm 0.06$	0.039
	DG(40:9)	$0.64 \times 10^5 \pm 0.10$	$0.31 \times 10^5 \pm 0.06$	0.039
Monoacylglycerols	MG(16:0)	$3.35 \times 10^5 \pm 0.28$	$1.94 \times 10^5 \pm 0.24$	0.044
	MG(18:0)	$0.48 \times 10^5 \pm 0.04$	$0.29 \times 10^5 \pm 0.03$	0.048
Fatty acyls	6-cis-docosenamide	$0.48 \times 10^5 \pm 0.04$	$0.29 \times 10^5 \pm 0.03$	0.048
	12,15-cis-squamostatin A	$1.00 \times 10^6 \pm 0.21$	$0.24 \times 10^6 \pm 0.05$	0.039
	Jetein	$5.71 \times 10^5 \pm 0.65$	$2.82 \times 10^5 \pm 0.73$	0.048
	1,2-dilinoleoyl-sn-glycerol	$4.43 \times 10^5 \pm 0.62$	$1.54 \times 10^5 \pm 0.41$	0.039
	disepalin	$4.04 \times 10^5 \pm 0.95$	$1.09 \times 10^5 \pm 0.26$	0.05
	12-hydroxy-9,10-dihydrojasmonic acid	$3.10 \times 10^5 \pm 0.24$	$1.94 \times 10^5 \pm 0.16$	0.041
	tonkinelin	$2.56 \times 10^5 \pm 0.34$	$1.22 \times 10^5 \pm 0.29$	0.048
	hydroxyphthioceranic acid	$1.76 \times 10^5 \pm 0.16$	$0.91 \times 10^5 \pm 0.19$	0.036
	cucurbic acid	$1.60 \times 10^5 \pm 0.18$	$0.90 \times 10^5 \pm 0.09$	0.05
	isoketocamphoric acid	$1.23 \times 10^5 \pm 0.12$	$0.49 \times 10^5 \pm 0.14$	0.041
	1-acetoxy-2-hydroxy-5,12,15-heneicosatrien-4-one	$0.98 \times 10^5 \pm 0.11$	$0.39 \times 10^5 \pm 0.07$	0.036
	type IV cyanolipid 20:0 ester	$0.85 \times 10^5 \pm 0.04$	$0.60 \times 10^5 \pm 0.06$	0.041
	prostaglandin D2-1-glyceryl ester	$0.79 \times 10^5 \pm 0.08$	$0.37 \times 10^5 \pm 0.05$	0.036
	(E,E,E)-N-(2-methylpropyl)hexadeca-2,6,8-trien-10-ynamide	$0.46 \times 10^5 \pm 0.05$	$0.23 \times 10^5 \pm 0.02$	0.039

	methyl 8-[3,5-epidioxy-2-(3-hydroperoxy-1-pentenyl)-cyclopentyl]-octanoate	$0.44 \times 10^5 \pm 0.02$	$0.26 \times 10^5 \pm 0.03$	0.036
	citramalic acid	$0.09 \times 10^5 \pm 0.001$	$0.03 \times 10^5 \pm 0.003$	0.036
Phospholipids	PS(31:0)	$3.94 \times 10^6 \pm 0.21$	$2.52 \times 10^6 \pm 0.27$	0.041
	PC(34:1)	$1.47 \times 10^6 \pm 0.21$	$0.84 \times 10^6 \pm 0.08$	0.039
	PC(36:2)	$0.96 \times 10^6 \pm 0.12$	$0.47 \times 10^6 \pm 0.07$	0.039
	PC(36:3)	$0.68 \times 10^6 \pm 0.07$	$0.34 \times 10^6 \pm 0.06$	0.039
	PS(36:4)	$0.35 \times 10^6 \pm 0.01$	$0.20 \times 10^6 \pm 0.02$	0.036
	PC(25:0)	$0.33 \times 10^6 \pm 0.03$	$0.20 \times 10^6 \pm 0.02$	0.048
	PC(36:1)	$0.30 \times 10^6 \pm 0.04$	$0.16 \times 10^6 \pm 0.02$	0.036
	PS(29:0)	$0.17 \times 10^6 \pm 0.02$	$0.09 \times 10^6 \pm 0.01$	0.036
	PE(38:1)	$0.10 \times 10^6 \pm 0.01$	$0.05 \times 10^6 \pm 0.004$	0.036
	1-archaetidyl-D-myo-inositol	$0.07 \times 10^6 \pm 0.005$	$0.04 \times 10^6 \pm 0.007$	0.039
	PS(37:3)	$0.06 \times 10^6 \pm 0.003$	$0.04 \times 10^6 \pm 0.004$	0.036
	PA(32:2)	$0.05 \times 10^6 \pm 0.01$	$0.01 \times 10^6 \pm 0.004$	0.039
	PS(30:1)	$0.05 \times 10^6 \pm 0.004$	$0.02 \times 10^6 \pm 0.003$	0.036
	PS(32:1)	$0.04 \times 10^6 \pm 0.007$	$0.01 \times 10^6 \pm 0.002$	0.039
	PE(38:4)	$0.04 \times 10^6 \pm 0.003$	$0.02 \times 10^6 \pm 0.004$	0.048
	PE(38:4)	$0.03 \times 10^6 \pm 0.004$	$0.02 \times 10^6 \pm 0.002$	0.041
	LPC(18:0)	$0.03 \times 10^6 \pm 0.004$	$0.01 \times 10^6 \pm 0.003$	0.048
	LPE(16:0)	$0.03 \times 10^6 \pm 0.001$	$0.02 \times 10^6 \pm 0.003$	0.039
	PS(29:0)	$0.03 \times 10^6 \pm 0.002$	$0.01 \times 10^6 \pm 0.003$	0.041
	PA(44:3)	$0.016 \times 10^6 \pm 0.003$	$0.005 \times 10^6 \pm 0.002$	0.041
	PA(46:4)	$0.008 \times 10^6 \pm 0.002$	$0.0008 \times 10^6 \pm 0.0001$	0.048
	PI(35:2)	$0.005 \times 10^6 \pm 0.0003$	$0.003 \times 10^6 \pm 0.0006$	0.048
Prenol lipids	geranylcitronellol	$6.22 \times 10^6 \pm 0.28$	$3.97 \times 10^6 \pm 0.68$	0.041
	3-(all-trans-npnaprenyl)benzene-1,2-diol	$1.14 \times 10^6 \pm 0.15$	$0.53 \times 10^6 \pm 0.09$	0.039
	menaquinol-11	$1.09 \times 10^6 \pm 0.12$	$0.57 \times 10^6 \pm 0.11$	0.048
	plastochromanol 8	$0.66 \times 10^6 \pm 0.08$	$0.33 \times 10^6 \pm 0.08$	0.039
	3-demethylubiquinol-10	$0.36 \times 10^6 \pm 0.05$	$0.14 \times 10^6 \pm 0.03$	0.045
	theasapogenol A	$0.27 \times 10^6 \pm 0.04$	$0.10 \times 10^6 \pm 0.01$	0.036
	14-deacetylnudicauline	$0.19 \times 10^6 \pm 0.03$	$0.05 \times 10^6 \pm 0.01$	0.039
	guayulin B	$0.18 \times 10^6 \pm 0.02$	$0.09 \times 10^6 \pm 0.01$	0.039
	Cassaidine	$0.18 \times 10^6 \pm 0.02$	$0.10 \times 10^6 \pm 0.02$	0.048
	reduced coenzyme Q10	$0.13 \times 10^6 \pm 0.01$	$0.06 \times 10^6 \pm 0.02$	0.048
	glisoprenin D	$0.12 \times 10^6 \pm 0.01$	$0.06 \times 10^6 \pm 0.01$	0.036
	3-epipapyriferic acid	$0.12 \times 10^6 \pm 0.02$	$0.05 \times 10^6 \pm 0.004$	0.048
	aplidiasphingosine	$0.11 \times 10^6 \pm 0.007$	$0.06 \times 10^6 \pm 0.007$	0.041
	solavetivone	$0.08 \times 10^6 \pm 0.01$	$0.03 \times 10^6 \pm 0.004$	0.044
	avadharidine	$0.03 \times 10^6 \pm 0.005$	$0.007 \times 10^6 \pm 0.001$	0.05
Sphingolipids	N-triacontanoyphytosphingosine	$7.80 \times 10^4 \pm 1.47$	$3.10 \times 10^4 \pm 0.66$	0.048
	SM(38:1)	$5.05 \times 10^4 \pm 0.63$	$3.72 \times 10^4 \pm 0.53$	0.049
	ganglioside GA2	$1.53 \times 10^4 \pm 0.13$	$1.07 \times 10^4 \pm 0.14$	0.041
Steroids	goyaglycoside c	$4.88 \times 10^6 \pm 0.21$	$3.18 \times 10^6 \pm 0.33$	0.039
	17-oxocycloprotobuxine	$2.89 \times 10^6 \pm 0.27$	$1.60 \times 10^6 \pm 0.22$	0.048
	ACGal C6	$0.12 \times 10^6 \pm 0.02$	$0.036 \times 10^6 \pm 0.006$	0.036
	momordicoside G	$0.12 \times 10^6 \pm 0.02$	$0.036 \times 10^6 \pm 0.005$	0.039
	sitoindoside I	$0.07 \times 10^6 \pm 0.006$	$0.033 \times 10^6 \pm 0.008$	0.041
	testosterone enanthate	$0.05 \times 10^6 \pm 0.002$	$0.033 \times 10^6 \pm 0.004$	0.036
	sulfolithocholic acid	$0.016 \times 10^6 \pm 0.001$	$0.009 \times 10^6 \pm 0.001$	0.041

Supplementary Table 3: Abundance of oocyte metabolites that were affected by supplementation with RSS2. Single oocytes were analyzed from the same mares Pre and Post approximately two months of supplementation. Results are presented as mean ± SEM.

Superclass	Metabolite	Pre	Post	P value
Alkaloids and derivatives	Pseudoconhydrine	$4.14 \times 10^4 \pm 0.30$	$2.66 \times 10^4 \pm 0.24$	0.022
	13alpha-(caproyloxy)lupanine	$3.71 \times 10^3 \pm 0.64$	$1.01 \times 10^3 \pm 0.27$	0.022
	17-O-acetyltetraphyllicine	$2.12 \times 10^4 \pm 0.43$	$0.37 \times 10^4 \pm 0.25$	0.034
	Jafrine	$3.70 \times 10^3 \pm 0.75$	$1.06 \times 10^3 \pm 0.13$	0.035
	Ecgonine methyl ester	$1.49 \times 10^5 \pm 0.30$	$0.42 \times 10^5 \pm 0.13$	0.042
	Strictosidine aglycone	$4.45 \times 10^4 \pm 0.54$	$2.52 \times 10^4 \pm 0.43$	0.064
	Prosopinine	$8.67 \times 10^3 \pm 0.85$	$5.84 \times 10^3 \pm 0.74$	0.09
	Tropinone	$1.05 \times 10^4 \pm 0.23$	$0.40 \times 10^4 \pm 0.13$	0.093
Benzenoids	Aristolochic acid III methyl ester	$1.37 \times 10^4 \pm 0.11$	$0.51 \times 10^4 \pm 0.07$	0.003
	6-[oxo-[2-[oxo-2-phenylethylamino)methyl]anilino] methyl]-1-cyclohex-3-enecarboxylis acid	$6.45 \times 10^2 \pm 0.16$	$3.89 \times 10^2 \pm 0.44$	0.009
	Methyl benzoate	$3.96 \times 10^3 \pm 0.31$	$2.10 \times 10^3 \pm 0.21$	0.012
	Grenadamide	$7.27 \times 10^4 \pm 0.89$	$2.95 \times 10^4 \pm 0.35$	0.016
	Fenpiprane	$2.23 \times 10^4 \pm 0.52$	$0.25 \times 10^4 \pm 0.05$	0.025
	Carbuterol	$1.14 \times 10^4 \pm 0.24$	$0.24 \times 10^6 \pm 0.06$	0.031
	(2S)-2-[[[4-[[4-[[[(2-methylpropan-2-yl)oxy-oxomethyl]amino]methyl]anilino]-oxomethyl]-1H-imidazol-5-yl]-oxomethyl]amino]propanoic acid tert-butyl ester	$1.96 \times 10^4 \pm 0.52$	$0.10 \times 10^4 \pm 0.05$	0.032
	Alprenolol	$1.55 \times 10^5 \pm 0.11$	$0.99 \times 10^5 \pm 0.13$	0.038
	Oxybuprocaine	$2.04 \times 10^4 \pm 0.30$	$4.91 \times 10^4 \pm 0.81$	0.040
	4-Allyl-2,6-dimethoxyphenol	$5.42 \times 10^4 \pm 0.21$	$3.98 \times 10^4 \pm 0.40$	0.044
	N-[3-(4-morpholinylsulfonyl)phenyl]-4-(2-oxo-1-pyrrolidinyl)benzamide	$0.68 \times 10^4 \pm 0.49$	$6.97 \times 10^4 \pm 1.95$	0.046
	Atenolol	$2.34 \times 10^5 \pm 0.16$	$1.58 \times 10^5 \pm 0.18$	0.048
	1-(3,5-dimethyl-4-isoxazolyl)-3-[(2S,3S)-5-[(2S)-1-hydroxypropan-2-yl]-2-[[(4-methoxyphenyl)methyl-methylamino]methyl]-3-methyl-6-oxo-3,4-dihydro-2H-1,5-benzoxazocin-8-yl]urea	$2.90 \times 10^4 \pm 0.76$	$0.62 \times 10^4 \pm 0.14$	0.056
	Aristolochic acid	$6.33 \times 10^3 \pm 0.11$	$2.92 \times 10^3 \pm 0.38$	0.062
	2-({[(4-methoxyphenyl)methyl](methyl)amino} methyl)-2-methylpropane-1,3-diol_2	$1.39 \times 10^4 \pm 0.47$	$0.16 \times 10^4 \pm 0.03$	0.080
	6-amino-4-(2-methoxyphenyl)-3-propyl-2,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	$5.05 \times 10^2 \pm 0.47$	$3.24 \times 10^2 \pm 0.53$	0.088
	trans-1-Phenyl-1-pentene	$2.20 \times 10^5 \pm 0.27$	$1.35 \times 10^5 \pm 0.20$	0.090
Lignans	Myricatomentoside I	$4.86 \times 10^4 \pm 0.13$	$3.09 \times 10^4 \pm 0.43$	0.021
Nucleosides	dTDP-5-dimethyl-L-lyxose	$4.68 \times 10^4 \pm 0.28$	$2.05 \times 10^4 \pm 0.21$	0.0007

Organic acids	Lotusanine B	$3.93 \times 10^5 \pm 0.26$	$1.56 \times 10^5 \pm 0.28$	0.003
	Thiazinotrienomycin G	$2.24 \times 10^4 \pm 0.01$	$1.51 \times 10^4 \pm 0.13$	0.016
	(2S)-2-[[(2R)-2-[(1S)-1-hydroxy-2-(hydroxyamino)-2-oxoethyl]-4-methyl-1-oxopentyl]amino]-2-phenylacetic acid cyclopentyl ester	$2.11 \times 10^4 \pm 0.01$	$1.37 \times 10^4 \pm 0.13$	0.016
	N2-Acetyl-L-aminoadipyl-delta-phosphate	$1.87 \times 10^4 \pm 0.13$	$1.09 \times 10^4 \pm 0.13$	0.019
	Restricticin	$2.34 \times 10^4 \pm 0.27$	$1.14 \times 10^4 \pm 0.12$	0.020
	D-Chicoric acid	$0.43 \times 10^4 \pm 0.16$	$2.12 \times 10^4 \pm 0.40$	0.022
	Haligramide B	$9.46 \times 10^3 \pm 0.51$	$6.77 \times 10^3 \pm 0.59$	0.036
	6-octenoylglycine	$1.22 \times 10^4 \pm 0.24$	$0.39 \times 10^4 \pm 0.06$	0.037
	Melagatran	$2.93 \times 10^4 \pm 0.84$	$0.16 \times 10^4 \pm 0.04$	0.040
	Ichthyotherminolide	$6.89 \times 10^4 \pm 1.75$	$1.14 \times 10^4 \pm 0.24$	0.041
	N-(2-Hydroxyethyl)decanamide	$1.17 \times 10^4 \pm 0.29$	$0.25 \times 10^4 \pm 0.05$	0.047
	Glutathione	$0.23 \times 10^5 \pm 0.15$	$1.14 \times 10^5 \pm 0.25$	0.048
	Ceanothine C	$1.90 \times 10^4 \pm 0.37$	$0.73 \times 10^4 \pm 0.11$	0.049
	Azotochelin	$0.82 \times 10^7 \pm 0.65$	$3.92 \times 10^7 \pm 0.79$	0.050
	GABA-stearamide	$3.05 \times 10^5 \pm 0.39$	$1.62 \times 10^5 \pm 0.27$	0.051
	N-decanoylglycine	$4.72 \times 10^4 \pm 0.31$	$3.24 \times 10^4 \pm 0.38$	0.053
	N-Butylacetamide	$5.60 \times 10^2 \pm 0.54$	$3.48 \times 10^2 \pm 0.47$	0.056
	3-(3,4-dimethoxyphenyl)-N-{2-[4-methoxy-3-(sulfooxy)phenyl]ethyl}prop-2-enimidic acid	$2.09 \times 10^6 \pm 1.11$	$7.35 \times 10^6 \pm 0.14$	0.057
	Cyclosquamosin G	$6.17 \times 10^3 \pm 1.53$	$1.56 \times 10^3 \pm 0.37$	0.057
	DES(2-methylbutanoyl)pravastatin	$1.10 \times 10^5 \pm 0.10$	$0.71 \times 10^5 \pm 0.10$	0.062
	3,12-dihydroxylaurate	$2.16 \times 10^5 \pm 0.30$	$1.17 \times 10^5 \pm 0.17$	0.063
	Sorbitan tristearate	$1.09 \times 10^5 \pm 0.10$	$0.60 \times 10^5 \pm 0.14$	0.064
	Melagatran	$2.14 \times 10^6 \pm 0.73$	$0.12 \times 10^6 \pm 0.04$	0.067
	D-2-Hydroxyglutaric acid	$2.45 \times 10^5 \pm 0.20$	$1.60 \times 10^5 \pm 0.24$	0.074
	Gabapentin enacarbil	$2.29 \times 10^4 \pm 0.82$	$0.10 \times 10^4 \pm 0.01$	0.077
	L-Theanine	$1.35 \times 10^5 \pm 0.07$	$1.05 \times 10^5 \pm 0.09$	0.077
	Leucylproline	$6.46 \times 10^3 \pm 1.64$	$2.07 \times 10^3 \pm 0.33$	0.079
	Tributyl phosphate	$5.31 \times 10^4 \pm 0.54$	$3.61 \times 10^4 \pm 0.39$	0.085
	methotrexate	$7.37 \times 10^3 \pm 0.55$	$4.85 \times 10^3 \pm 0.83$	0.088
	2-(3'-Methylthio)propylmalic acid	$1.79 \times 10^5 \pm 0.19$	$1.23 \times 10^5 \pm 0.13$	0.093
	Phaseolic acid	$8.04 \times 10^4 \pm 0.95$	$4.38 \times 10^4 \pm 1.13$	0.093
	(Z)-N-hydroxy-11-methyldodec-2-enamide	$2.77 \times 10^6 \pm 0.69$	$0.99 \times 10^6 \pm 0.19$	0.093
	Hexacosanoic acid isobutylamide	$5.29 \times 10^3 \pm 0.70$	$3.11 \times 10^3 \pm 0.52$	0.093
	GABA-stearamide	$3.57 \times 10^4 \pm 0.81$	$1.50 \times 10^4 \pm 0.25$	0.098
Organic nitrogen compounds	Linoleoyl ethanolamide	$3.19 \times 10^4 \pm 0.32$	$0.98 \times 10^4 \pm 0.17$	0.004
	1-hexadecyl-2-ammonio-2-deoxy-sn-glycerol	$1.20 \times 10^5 \pm 0.28$	$0.75 \times 10^5 \pm 0.08$	0.010
	15-methylhexadecasphinganine	$6.74 \times 10^5 \pm 0.27$	$4.32 \times 10^5 \pm 0.41$	0.013
	N-(2-hydroxyheptacosanoyl)-4-hydroxy-15-methylhexadecasphinganine-1-phosphocholine	$4.80 \times 10^5 \pm 0.61$	$1.94 \times 10^5 \pm 0.48$	0.028
	N-icosanoyl-4-hydroxy-15-methylhexadecasphinganine-1-phosphocholine	$1.48 \times 10^5 \pm 0.13$	$0.77 \times 10^5 \pm 0.17$	0.037

	n-oleoylethanolamine	$1.59 \times 10^5 \pm 0.17$	$0.93 \times 10^5 \pm 0.12$	0.045
	Dioctylamine	$5.44 \times 10^5 \pm 0.45$	$3.58 \times 10^5 \pm 0.40$	0.047
	Halaminol A	$1.10 \times 10^5 \pm 0.09$	$0.70 \times 10^5 \pm 0.09$	0.047
	Octamylamine	$6.73 \times 10^4 \pm 0.66$	$4.08 \times 10^4 \pm 0.59$	0.053
	Octylamine	$4.71 \times 10^5 \pm 0.77$	$2.39 \times 10^5 \pm 0.26$	0.062
	N-gondoylethanolamine	$2.13 \times 10^6 \pm 0.32$	$1.12 \times 10^6 \pm 0.16$	0.062
	N-(2-hydroxypentacosanoyl)-15-methylhexadecasphing-4-enine-1-phosphocholine	$1.47 \times 10^5 \pm 0.20$	$0.77 \times 10^5 \pm 0.14$	0.063
	N-(11Z,14Z)-eicosadienoylethanolamine	$3.14 \times 10^5 \pm 0.36$	$1.80 \times 10^5 \pm 0.31$	0.063
	2-(5-Methyl-2-furanyl)-3-piperidinol	$1.07 \times 10^4 \pm 0.19$	$0.49 \times 10^4 \pm 0.07$	0.066
	Octodrine	$9.92 \times 10^4 \pm 3.21$	$1.48 \times 10^4 \pm 1.01$	0.090
Organic oxygen compounds	2-[(2R,4aR,12aS)-5-methyl-6-oxo-8-[[oxo-(propan-2-ylamino)methyl]amino]-2,3,4,4a,12,12a-hexahydropyrano[2,3-c][1,5]benzoxazocin-2-yl]-N-propylacetamide	$5.26 \times 10^4 \pm 0.35$	$1.61 \times 10^4 \pm 0.25$	0.0005
	Dimethyl 2-galloylgalactarate	$9.67 \times 10^4 \pm 0.73$	$3.03 \times 10^4 \pm 0.37$	0.0005
	Fluvirucin B2	$4.49 \times 10^4 \pm 0.23$	$2.79 \times 10^4 \pm 0.26$	0.013
	Pentaethylene glycol monododecyl ether	$1.54 \times 10^5 \pm 0.06$	$0.99 \times 10^5 \pm 0.10$	0.015
	Fortimicin KL1	$4.95 \times 10^3 \pm 0.54$	$1.85 \times 10^3 \pm 0.47$	0.016
	6-[1-carboxy-2-(4-methoxy-1-benzofuran-5-yl)-2-oxoethyl]-3,4,5-trihydroxyoxane-2-carboxylic acid	$2.51 \times 10^5 \pm 0.22$	$1.22 \times 10^5 \pm 0.21$	0.018
	Alpha-Butyl-omega-hydroxypoly(oxyethylene) poly(oxypropylene)	$5.98 \times 10^4 \pm 0.56$	$3.26 \times 10^4 \pm 0.31$	0.018
	Midecamycin acetate	$6.37 \times 10^2 \pm 0.46$	$3.30 \times 10^2 \pm 0.58$	0.020
	Itoside K	$5.46 \times 10^4 \pm 0.67$	$2.44 \times 10^4 \pm 0.26$	0.020
	6-[5-(1-carboxyethyl)-4-hydroxy-2-methoxyphenoxy]-3,4,5-trihydroxyoxane-2-carboxylic acid	$4.85 \times 10^5 \pm 0.32$	$2.84 \times 10^5 \pm 0.39$	0.021
	Picrocrocin	$7.47 \times 10^5 \pm 1.22$	$2.37 \times 10^5 \pm 0.71$	0.030
	5-decanoyl-2-nonylpyridine	$3.79 \times 10^4 \pm 0.26$	$2.37 \times 10^4 \pm 0.32$	0.036
	N'-monoacetylchitobiose-6'-phosphate(1-)	$1.13 \times 10^5 \pm 0.12$	$0.65 \times 10^5 \pm 0.08$	0.041
	Ciramadol	$2.19 \times 10^6 \pm 0.28$	$1.13 \times 10^6 \pm 0.16$	0.041
	N-[(2R,3R,6R)-2-(hydroxymethyl)-6-[2-oxo-2-[2-(1-piperidinyl)ethylamino]ethyl]-3-oxanyl]propanamide	$1.46 \times 10^4 \pm 0.28$	$2.79 \times 10^4 \pm 0.32$	0.045
	N-(2-hydroxyheptacosanoyl)-15-methylhexadecasphing-4-enine	$1.28 \times 10^5 \pm 0.20$	$0.60 \times 10^5 \pm 0.10$	0.054
	3,6,9,12,15-Pentaoxaheptadecane	$1.02 \times 10^5 \pm 0.17$	$0.65 \times 10^5 \pm 0.06$	0.062
	8,8-Diethoxy-2,6-dimethyl-2-octanol	$2.77 \times 10^4 \pm 0.15$	$1.93 \times 10^4 \pm 0.25$	0.062
	(N-acetylneuraminosyl(a2-6)lactosamine)	$6.27 \times 10^5 \pm 0.34$	$4.59 \times 10^5 \pm 0.49$	0.064
	Scabran G3	$7.21 \times 10^5 \pm 0.47$	$5.40 \times 10^5 \pm 0.47$	0.069
	N,O-Didesmethylvenlafaxine	$2.06 \times 10^5 \pm 0.18$	$1.34 \times 10^5 \pm 0.20$	0.072
	4-hydroxybenzaldehyde	$2.12 \times 10^4 \pm 0.21$	$1.44 \times 10^4 \pm 0.15$	0.076
	Sorgoleone 358	$1.83 \times 10^4 \pm 0.17$	$1.21 \times 10^4 \pm 0.17$	0.082

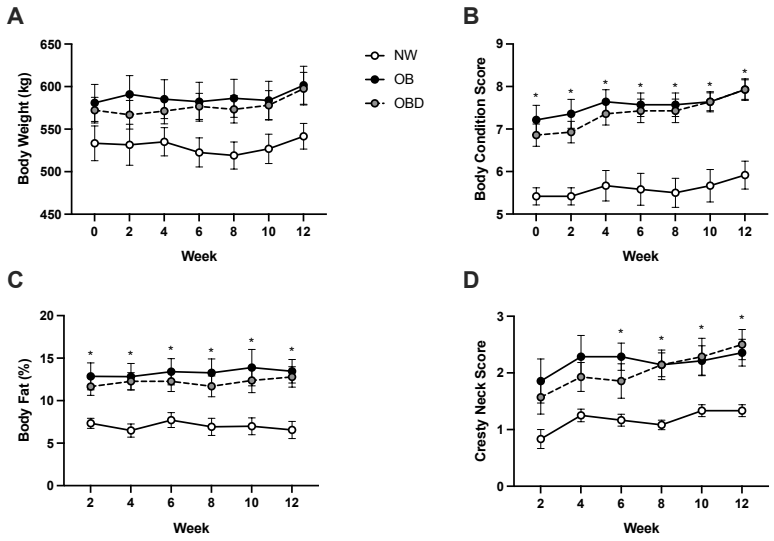
	Glucocamelinin	$5.26 \times 10^3 \pm 1.02$	$2.43 \times 10^3 \pm 0.45$	0.088
	(2E,4E)-Octa-2,4-dienal	$9.72 \times 10^2 \pm 1.03$	$6.30 \times 10^2 \pm 0.95$	0.097
	2,4-Dihydroxyacetophenone 5-sulfate	$3.69 \times 10^3 \pm 1.00$	$1.24 \times 10^3 \pm 0.11$	0.098
	N-octacosanoyl-4-hydroxy-15-methylhexadecasphinganine	$4.85 \times 10^4 \pm 0.91$	$2.30 \times 10^4 \pm 0.52$	0.099
	N-(2-hydroxytetracosanoyl)-15-methylhexadecasphing-4-enine	$6.82 \times 10^4 \pm 1.37$	$3.16 \times 10^4 \pm 0.70$	0.099
Organoheterocyclic compounds	trans-2-Butyl-5-pentylpyrrolidine	$6.04 \times 10^4 \pm 0.75$	$2.30 \times 10^4 \pm 0.26$	0.014
	5,8-Dihydro-6-(4-methyl-3-pentenyl)-1,2,3,4-tetrathiocin	$3.13 \times 10^4 \pm 0.28$	$1.45 \times 10^4 \pm 0.25$	0.016
	2,5-dimethylfuran	$1.09 \times 10^3 \pm 0.10$	$0.53 \times 10^3 \pm 0.07$	0.016
	Austinol	$3.49 \times 10^4 \pm 0.14$	$2.26 \times 10^4 \pm 0.26$	0.018
	2-carboxy-5,7-dimethyl-4-octanolide	$1.60 \times 10^5 \pm 0.18$	$0.86 \times 10^5 \pm 0.05$	0.021
	5-(1-methyl-2-benzimidazolyl)-2-thiophenecarboxaldehyde	$2.23 \times 10^4 \pm 0.34$	$0.91 \times 10^4 \pm 0.07$	0.025
	Arachidonoylmorpholine	$3.67 \times 10^4 \pm 0.28$	$2.19 \times 10^4 \pm 0.31$	0.030
	2-isopropyl-5-methylfuran	$7.71 \times 10^3 \pm 0.71$	$4.25 \times 10^3 \pm 0.65$	0.030
	Iproniazid	$1.62 \times 10^3 \pm 0.10$	$1.12 \times 10^3 \pm 0.10$	0.036
	(6R,7S,8aS)-N-(5-aminopentyl)-6-(4-hydroxyphenyl)-1,4-dioxo-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-7-carboxamide	$2.01 \times 10^5 \pm 0.18$	$1.28 \times 10^5 \pm 0.12$	0.037
	2-hexyl-4-methyl-5-ethyloxazole	$4.42 \times 10^3 \pm 0.88$	$1.23 \times 10^3 \pm 0.41$	0.041
	Famotidine	$9.80 \times 10^4 \pm 0.69$	$6.29 \times 10^4 \pm 0.84$	0.043
	1-hexadecanoylpyrrolidine	$5.60 \times 10^4 \pm 0.55$	$3.31 \times 10^4 \pm 0.47$	0.045
	2-cyclopropyl-1-[(2S,3R)-2-(hydroxymethyl)-3-phenyl-6-(3-pyridinylmethyl)-1,6-diazaspiro[3.3]heptan-1-yl]ethanone	$2.15 \times 10^4 \pm 0.19$	$1.20 \times 10^4 \pm 0.24$	0.047
	4-methoxy-N-[2-[(4-nitrophenyl)methylthio]-4-oxo-3-quinazolinyl]benzamide	$0.35 \times 10^6 \pm 0.23$	$2.76 \times 10^6 \pm 0.74$	0.047
	Cetilistat	$6.74 \times 10^5 \pm 0.60$	$3.99 \times 10^5 \pm 0.66$	0.047
	4-[(3aR,4R,9bR)-1-[cyclohexyl(oxo)methyl]-4-(hydroxymethyl)-2,3,3a,4,5,9b-hexahydropyrrolo[3,2-c]quinolin-8-yl]benzonitrile	$1.04 \times 10^5 \pm 0.27$	$0.19 \times 10^5 \pm 0.07$	0.049
	1-butyl-5-[1-[2-(1H-indol-3-yl)ethylamino]ethylidene]-1,3-diazinane-2,4,6-trione	$2.53 \times 10^3 \pm 0.37$	$1.17 \times 10^3 \pm 0.26$	0.051
	Pyrinodemin D	$1.11 \times 10^5 \pm 0.08$	$0.76 \times 10^5 \pm 0.09$	0.052
	Brassicanal B	$0.38 \times 10^6 \pm 0.22$	$1.78 \times 10^6 \pm 0.43$	0.060
	7-[2-hydroxy-3-(4-methoxyphenoxy)propyl]-1,3-dimethyl-8-(1-piperidinyl)purine-2,6-dione	$4.67 \times 10^4 \pm 1.61$	$0.10 \times 10^4 \pm 0.04$	0.062
	Ditalimfos	$0.79 \times 10^5 \pm 0.44$	$3.37 \times 10^5 \pm 0.80$	0.063
	Phendimetrazine	$7.26 \times 10^4 \pm 0.53$	$5.20 \times 10^4 \pm 0.51$	0.064
	Brachyamide B	$1.73 \times 10^4 \pm 0.38$	$0.61 \times 10^4 \pm 0.16$	0.071
	1,2,3,4,5,6-hexahydro-5-(1-hydroxyethylidene)-7H-cyclopenta[b]pyridin-7-one	$1.20 \times 10^4 \pm 0.28$	$0.33 \times 10^4 \pm 0.15$	0.072
	1-ethylpiperidine	$2.16 \times 10^3 \pm 0.48$	$0.65 \times 10^3 \pm 0.30$	0.073
	6,7-Epoxy-3Z,9Z-tricosadiene	$4.73 \times 10^5 \pm 0.37$	$2.95 \times 10^5 \pm 0.55$	0.075

	Indole-3-carbinol	$6.00 \times 10^3 \pm 1.43$	$2.11 \times 10^3 \pm 0.32$	0.077
	Scorodocarpine C	$2.73 \times 10^4 \pm 0.32$	$1.76 \times 10^4 \pm 0.18$	0.077
	Hapovine	$1.78 \times 10^3 \pm 0.26$	$0.93 \times 10^3 \pm 0.20$	0.079
	3-Isopropyl-2-methoxy-5-methylpyrazine	$9.91 \times 10^4 \pm 1.35$	$5.77 \times 10^4 \pm 0.87$	0.084
	Azepan-2-one	$1.03 \times 10^6 \pm 0.24$	$0.40 \times 10^6 \pm 0.05$	0.085
	N-[(Z)-dodec-2-enoyl]morpholine	$3.96 \times 10^3 \pm 1.12$	$1.00 \times 10^3 \pm 0.32$	0.088
	Phomacin B	$2.11 \times 10^4 \pm 0.24$	$1.33 \times 10^4 \pm 0.21$	0.093
	2-[[3-(4-morpholinylmethyl)-2H-1-benzopyran-8-yl]oxymethyl]morpholine	$4.73 \times 10^3 \pm 1.30$	$1.39 \times 10^3 \pm 0.37$	0.094
	Pipercallosidine	$2.33 \times 10^3 \pm 0.61$	$0.77 \times 10^3 \pm 0.20$	0.097
	Sieboldine A	$1.17 \times 10^6 \pm 0.14$	$0.71 \times 10^6 \pm 0.12$	0.099
Phenylpropanoids and polyketides	3-(2,4-dihydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid	$1.21 \times 10^5 \pm 0.10$	$0.41 \times 10^5 \pm 0.05$	0.0008
	Cytotrienin A	$3.21 \times 10^5 \pm 0.32$	$0.52 \times 10^4 \pm 0.16$	0.004
	Feruloylagmatine	$2.13 \times 10^4 \pm 0.25$	$1.22 \times 10^4 \pm 0.19$	0.009
	1,2,6-trigalloyl-beta-D-glucopyranose	$3.06 \times 10^4 \pm 0.19$	$1.51 \times 10^4 \pm 0.24$	0.011
	{5-[2,3-dioxo-3-(2,4,6-trihydroxy-3-methoxyphenyl)propyl]-2-hydroxyphenyl}oxidanesulfonic acid	$8.67 \times 10^3 \pm 1.43$	$1.75 \times 10^3 \pm 0.51$	0.015
	Pinocembrin 7-O-neohesperidoside 6''-O-acetate	$3.03 \times 10^5 \pm 0.17$	$1.65 \times 10^5 \pm 0.25$	0.015
	8-hydroxyluteolin 7-[6'''-acetylallosyl-(1->2)-glucoside]	$2.10 \times 10^4 \pm 0.12$	$1.35 \times 10^4 \pm 0.13$	0.018
	4-[1-ethyl-2-(4-methylphenyl)butyl]phenol	$2.29 \times 10^3 \pm 0.43$	$0.52 \times 10^3 \pm 0.14$	0.021
	3-[3,4-dihydroxy-5-(3,4,5-trihydroxybenzoyloxy)benzoyloxy]-5-hydroxy-4-methoxybenzoic acid	$3.91 \times 10^4 \pm 0.42$	$2.04 \times 10^4 \pm 0.21$	0.021
	[10-butanoyl-5-hydroxy-6-(2-hydroxypropyl)-2,2-dimethyl-8-oxo-2H,3H,4H,8H-pyrano[3,2-g]chromen-3-yl]oxidanesulfonic acid	$3.32 \times 10^3 \pm 0.27$	$1.90 \times 10^3 \pm 0.24$	0.022
	N-[[(8R,9R)-6-[(2S)-1-hydroxypropan-2-yl]-8-methyl-5-oxo-10-oxa-1,6,14,15-tetrazabicyclo[10.3.0]pentadeca-12,14-dien-9-yl]methyl]-N-methylcyclopropanecarboxamide	$2.76 \times 10^5 \pm 0.72$	$0.07 \times 10^5 \pm 0.02$	0.027
	N-[(3R,9S,10S)-12-[(2R)-1-hydroxypropan-2-yl]-3,10-dimethyl-9-[[methyl(propyl)amino]methyl]-13-oxo-2,8-dioxa-12-azabicyclo[12.4.0]octadeca-1(14),15,17-trien-16-yl]-4-pyridinecarboxamide	$1.75 \times 10^5 \pm 0.37$	$0.39 \times 10^5 \pm 0.06$	0.030
	2-hydroxy-24-keto-octacosanolide	$2.35 \times 10^5 \pm 0.18$	$1.50 \times 10^5 \pm 0.15$	0.031
	3-[4,10'-epoxylinalyl]-5-methyl coumarin	$1.15 \times 10^4 \pm 0.21$	$0.32 \times 10^4 \pm 0.12$	0.036
	5-hydroxy-6-methoxycoumarin 7-glucoside	$1.09 \times 10^5 \pm 0.10$	$0.68 \times 10^5 \pm 0.06$	0.036
	Rhamnetin	$1.91 \times 10^5 \pm 0.34$	$0.76 \times 10^5 \pm 0.07$	0.041
	(3S,3'S,4'R,6'S,8'R,8'aR)-5-[3-(carbamoylamino)prop-1-ynyl]-6'-[4-(2-hydroxyethoxy)phenyl]-1',2-dioxo-	$5.06 \times 10^3 \pm 0.33$	$3.45 \times 10^3 \pm 0.36$	0.041

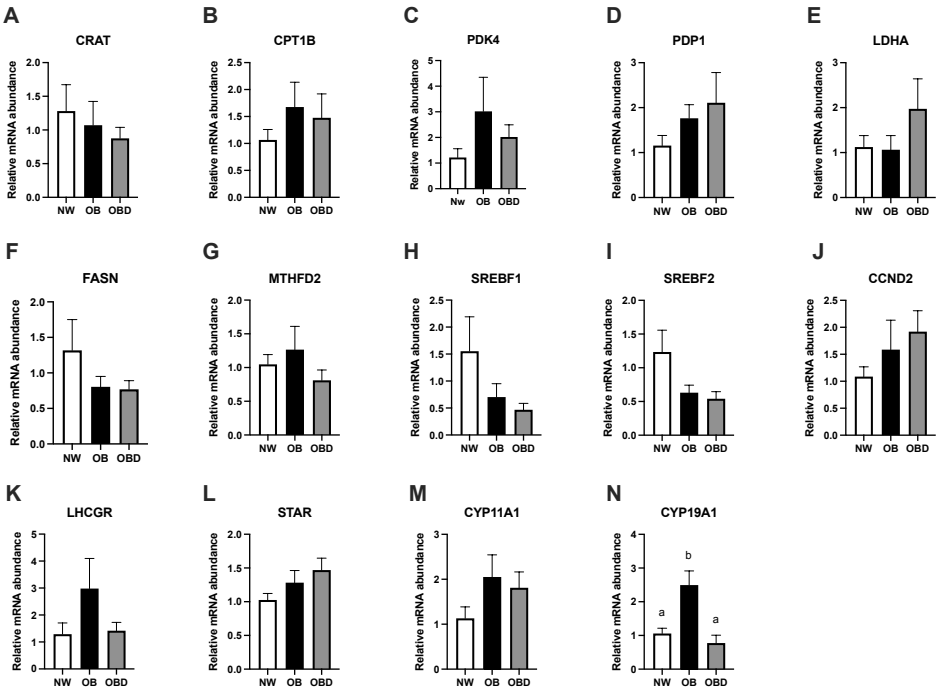
3',4'-diphenyl-N-prop-2-enyl-8'-spiro[1H-indole-3,7'-4,6,8,8a-tetrahydro-3H-pyrrolo[2,1-c][1,4]oxazine]carboxamide			
2-cinnamoyl-1,6-digalloyl-beta-D-glucopyranose	$1.25 \times 10^5 \pm 0.13$	$0.75 \times 10^5 \pm 0.09$	0.045
Kadsuphilol F	$1.15 \times 10^5 \pm 0.09$	$0.78 \times 10^5 \pm 0.08$	0.047
Acacetin 7-(4'''-acetylrutinoside)	$1.90 \times 10^4 \pm 0.19$	$1.20 \times 10^4 \pm 0.12$	0.047
Chakaflavonoside A	$1.22 \times 10^4 \pm 0.11$	$0.81 \times 10^4 \pm 0.81$	0.048
Volkensiflavone	$5.00 \times 10^4 \pm 0.39$	$3.16 \times 10^4 \pm 0.48$	0.052
Epothilone C6	$8.11 \times 10^4 \pm 2.04$	$1.86 \times 10^4 \pm 0.58$	0.055
Persicarin	$8.09 \times 10^4 \pm 2.65$	$0.64 \times 10^4 \pm 0.34$	0.064
2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-7-{[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4H-chromen-4-one	$1.30 \times 10^5 \pm 0.14$	$0.73 \times 10^5 \pm 0.15$	0.070
Hesperetin 7-glucoside	$8.34 \times 10^3 \pm 0.73$	$5.45 \times 10^3 \pm 0.81$	0.077
curcumin 4',4''-O-D-digentiobioside	$4.67 \times 10^3 \pm 0.50$	$3.03 \times 10^3 \pm 0.38$	0.079
3',6-disinapoylsucrose	$8.22 \times 10^5 \pm 0.51$	$6.40 \times 10^5 \pm 0.49$	0.084
Rhoifolin	$8.18 \times 10^3 \pm 1.26$	$4.52 \times 10^3 \pm 0.77$	0.084
Palmerolide A	$1.29 \times 10^5 \pm 0.18$	$0.75 \times 10^5 \pm 0.13$	0.098
3-(1,1-dimethylallyl)scopoletin 7-glucoside	$1.85 \times 10^3 \pm 0.34$	$0.93 \times 10^3 \pm 0.16$	0.098

APPENDIX III

Supplemental material Chapter IV: Follicular metabolic alterations associated with obesity in mares can be mitigated by dietary supplementation.



Supplementary Figure 2: Morphometric measurements of mares. Morphometric measurements from normal weight (NW, n=6), obese (OB, n=7) and obese diet supplemented (ODB, n=7) mares were performed at 2-week intervals: (**A**) body weight in kg, (**B**) body condition score (scored from 1, emaciated to 9, extreme obesity), (**C**) percentage of body fat, and (**D**) cresty neck score (0, none to 5, large crest dropped to one side). Graphs represent mean ± SEM. Asterisks denote differences in a given week between NW and obese groups (OB and OBD) using two-way ANOVA ($P<0.05$).



Supplementary Figure 3: Effects of mare obesity and diet supplementation on granulosa cell gene expression. Expression of genes of interest in granulosa cells obtained from preovulatory follicles of normal-weight (NW, n=6), obese (OB, n=7) and obese diet supplemented (OBD, n=6) mares after ≥ 6 weeks of supplementation. Data were normalized to expression of a housekeeping gene (*GAPDH*: glyceraldehyde 3-phosphate dehydrogenase) and are presented as fold changes relative to the control group (NW): (A) *CRAT*: carnitine acetyltransferase, (B) *CPT1B:* carnitine palmitoyltransferase IB, (C) *PDK4:* pyruvate dehydrogenase kinase 4, (D) *PDP1:* pyruvate dehydrogenase, (E) *LDHA:* lactate dehydrogenase A, (F) *FASN:* fatty acid synthase, (G) *MTHFD2:* methylenetetrahydrofolate dehydrogenase 2, (H) *SREBF1:* sterol regulatory element-binding protein1, (I) *SREBF2:* sterol regulatory element-binding protein 2, (J) *CCND2:* cyclin D2, (K) *LHCGR:* luteinizing hormone receptor, (L) *STAR:* steroidogenic acute regulatory protein, (M) *CYP11A1:* cholesterol side-chain cleavage enzyme, and (N) *CYP19A1:* aromatase. Graphs represent mean ± SEM. Different superscripts indicate difference (^{ab}, $P<0.05$) between groups using one-way ANOVA with post-hoc Tukey's multiple comparison tests, or Kruskal-Wallis tests, followed by Dunn's multiple comparison tests.

Supplementary Table 4: Abundance of lipids in cumulus cells that differed between groups (NW; n=5, OB, n=7; OBD, n=6). Results are presented as mean ± SEM. Different superscripts within the same row indicate difference (^{ab}, $P < 0.05$) or tendency for difference (^{cd}, $P < 0.1$) between groups using one-way ANOVA with post-hoc Tukey's multiple comparison tests.

Lipid species	NW	OB	OBD	P value (NW x OB)	P value (NW x OBD)	P value (OB x OBD)
TG(62:5)	$0.22 \times 10^5 \pm 0.11^{c}$	$1.31 \times 10^5 \pm 0.40^{d}$	$0.46 \times 10^5 \pm 0.12^{cd}$	0.06	0.8	0.2

TG(58:4)	$1.57 \times 10^5 \pm 0.67^{c}$	$5.70 \times 10^5 \pm 1.31^{d}$	$3.35 \times 10^5 \pm 0.70^{cd}$	0.08	0.5	0.5
TG(60:3)	$0.21 \times 10^5 \pm 0.09^{ac}$	$1.25 \times 10^5 \pm 0.33^{bd}$	$0.36 \times 10^5 \pm 0.12^{c}$	0.03	0.9	0.09
TG(60:4)	$0.32 \times 10^5 \pm 0.15^{a}$	$1.71 \times 10^5 \pm 0.48^{b}$	$0.65 \times 10^5 \pm 0.18^{ab}$	0.05	0.8	0.2
TG(68:7)	$0.97 \times 10^4 \pm 0.42^{c}$	$6.40 \times 10^4 \pm 2.07^{d}$	$2.15 \times 10^4 \pm 0.32^{cd}$	0.06	0.9	0.2
TG(54:2)	$1.30 \times 10^6 \pm 0.47^{c}$	$3.74 \times 10^6 \pm 0.77^{d}$	$2.17 \times 10^6 \pm 0.26^{cd}$	0.08	0.6	0.4
TG(54:1)	$1.79 \times 10^5 \pm 0.58^{a}$	$5.90 \times 10^5 \pm 1.01^{b}$	$2.94 \times 10^5 \pm 0.45^{ab}$	0.02	0.6	0.1
TG(56:3)	$0.44 \times 10^6 \pm 0.18^{c}$	$1.33 \times 10^6 \pm 0.27^{d}$	$0.83 \times 10^6 \pm 0.15^{cd}$	0.09	0.5	0.5
TG(58:3)	$0.73 \times 10^5 \pm 0.33^{c}$	$3.27 \times 10^5 \pm 0.88^{d}$	$1.44 \times 10^5 \pm 0.37^{cd}$	0.07	0.7	0.3
TG(60:2)	$1.20 \times 10^4 \pm 0.32^{a}$	$5.22 \times 10^4 \pm 1.19^{b}$	$1.37 \times 10^4 \pm 0.34^{a}$	0.02	0.97	0.03
TG(50:1)	$0.92 \times 10^6 \pm 0.19^{c}$	$1.72 \times 10^6 \pm 0.22^{d}$	$1.17 \times 10^6 \pm 0.08^{cd}$	0.08	0.6	0.4
TG(56:2)	$1.04 \times 10^5 \pm 0.42^{c}$	$3.93 \times 10^5 \pm 0.88^{d}$	$2.05 \times 10^5 \pm 0.55^{cd}$	0.06	0.6	0.3
TG(50:2)	$1.53 \times 10^6 \pm 0.40^{c}$	$3.22 \times 10^6 \pm 0.45^{d}$	$2.32 \times 10^6 \pm 0.25^{cd}$	0.09	0.4	0.6
TG(60:5)	$0.89 \times 10^5 \pm 0.40^{c}$	$4.06 \times 10^5 \pm 1.17^{d}$	$2.16 \times 10^5 \pm 0.50^{cd}$	0.09	0.6	0.5
TG(58:2)	$0.31 \times 10^5 \pm 0.10^{ac}$	$1.48 \times 10^5 \pm 0.34^{bd}$	$0.47 \times 10^5 \pm 0.13^{c}$	0.02	0.9	0.06
TG(62:4)	$1.40 \times 10^4 \pm 0.76^{a}$	$8.86 \times 10^4 \pm 2.66^{b}$	$2.61 \times 10^4 \pm 0.70^{ab}$	0.05	0.9	0.1
TG(54:5)	$0.40 \times 10^4 \pm 0.12^{a}$	$1.88 \times 10^4 \pm 0.54^{b}$	$0.69 \times 10^4 \pm 0.07^{ab}$	0.04	0.8	0.1
TG(48:3)	$2.94 \times 10^5 \pm 0.79^{c}$	$8.68 \times 10^5 \pm 0.13^{d}$	$5.17 \times 10^5 \pm 0.76^{cd}$	0.09	0.7	0.3
TG(52:1)	$0.99 \times 10^6 \pm 0.32^{c}$	$2.55 \times 10^6 \pm 0.51^{d}$	$1.58 \times 10^6 \pm 0.18^{cd}$	0.09	0.6	0.5
TG(52:0)	$2.55 \times 10^5 \pm 0.55^{a}$	$5.09 \times 10^5 \pm 0.68^{b}$	$3.53 \times 10^5 \pm 0.16^{ab}$	0.02	0.4	0.2
TG(64:5)	$0.62 \times 10^4 \pm 0.33^{a}$	$3.93 \times 10^4 \pm 1.17^{b}$	$1.24 \times 10^4 \pm 0.34^{ab}$	0.05	0.8	0.1
TG(56:1)	$0.38 \times 10^5 \pm 0.08^{a}$	$1.47 \times 10^5 \pm 0.27^{b}$	$0.49 \times 10^5 \pm 0.07^{a}$	0.008	0.9	0.02
TG(66:4)	$0.75 \times 10^4 \pm 0.08^{c}$	$1.29 \times 10^4 \pm 0.21^{d}$	$0.85 \times 10^4 \pm 0.05^{cd}$	0.07	0.8	0.2
TG(58:10)	$0.35 \times 10^5 \pm 0.09^{a}$	$1.17 \times 10^5 \pm 0.16^{b}$	$0.45 \times 10^5 \pm 0.05^{a}$	0.005	0.8	0.02
TG(50:3)	$1.03 \times 10^4 \pm 0.14^{c}$	$1.69 \times 10^4 \pm 0.14^{d}$	$1.15 \times 10^4 \pm 0.06^{cd}$	0.08	0.9	0.2
TG(52:4)	$0.23 \times 10^4 \pm 0.09^{c}$	$1.01 \times 10^4 \pm 0.33^{d}$	$0.32 \times 10^4 \pm 0.03^{cd}$	0.09	0.97	0.1
TG(55:3)	$1.62 \times 10^3 \pm 0.23^{a}$	$6.27 \times 10^3 \pm 1.26^{b}$	$3.32 \times 10^3 \pm 0.85^{ab}$	0.05	0.6	0.3
TG(54:4)	$2.51 \times 10^3 \pm 0.40^{c}$	$6.55 \times 10^3 \pm 1.34^{d}$	$3.74 \times 10^3 \pm 0.42^{cd}$	0.06	0.7	0.2

TG(64:4)	$0.40 \times 10^4 \pm 0.19^{c}$	$2.22 \times 10^4 \pm 0.72^{d}$	$0.76 \times 10^4 \pm 0.24^{cd}$	0.07	0.8	0.2
TG(50:1)	$1.31 \times 10^4 \pm 0.48^{c}$	$3.43 \times 10^4 \pm 0.73^{d}$	$2.22 \times 10^4 \pm 0.22^{cd}$	0.09	0.5	0.5
TG(54:3)	$6.29 \times 10^4 \pm 0.79^{c}$	$3.71 \times 10^4 \pm 0.59^{d}$	$4.74 \times 10^4 \pm 0.60^{cd}$	0.09	0.3	0.8
TG(60:11)	$3.32 \times 10^3 \pm 0.58^{a}$	$8.99 \times 10^3 \pm 1.20^{b}$	$7.17 \times 10^3 \pm 1.02^{ab}$	0.05	0.2	0.8
TG(54:5)	$2.43 \times 10^3 \pm 0.43^{a}$	$7.79 \times 10^3 \pm 1.11^{b}$	$4.25 \times 10^3 \pm 0.48^{ab}$	0.03	0.7	0.2
TG(61:4)	$0.22 \times 10^4 \pm 0.10^{c}$	$1.44 \times 10^4 \pm 0.45^{d}$	$0.43 \times 10^4 \pm 0.17^{cd}$	0.06	0.9	0.1
TG(53:1)	$0.39 \times 10^4 \pm 0.06^{a}$	$1.68 \times 10^4 \pm 0.43^{b}$	$0.46 \times 10^4 \pm 0.09^{a}$	0.02	0.97	0.04
TG(50:2)	$0.88 \times 10^5 \pm 0.21^{a}$	$1.77 \times 10^5 \pm 0.10^{b}$	$1.15 \times 10^5 \pm 0.11^{ab}$	0.04	0.6	0.2
TG(50:2)	$0.91 \times 10^4 \pm 0.26^{c}$	$2.31 \times 10^4 \pm 0.29^{d}$	$1.85 \times 10^4 \pm 0.18^{cd}$	0.08	0.2	0.9
TG(58:10)	$1.03 \times 10^4 \pm 0.14^{ab}$	$1.13 \times 10^4 \pm 0.23^{a}$	$0.50 \times 10^4 \pm 0.17^{b}$	0.8	0.2	0.04
DG(36:1)	$3.83 \times 10^4 \pm 0.74^{a}$	$9.73 \times 10^4 \pm 1.58^{b}$	$5.65 \times 10^4 \pm 0.31^{ab}$	0.02	0.5	0.1
DG(36:1)	$0.38 \times 10^5 \pm 0.12^{a}$	$1.23 \times 10^5 \pm 0.23^{b}$	$0.71 \times 10^5 \pm 0.08^{ab}$	0.04	0.5	0.3
DG(38:5)	$6.39 \times 10^2 \pm 0.54^{c}$	$7.26 \times 10^2 \pm 0.90^{cd}$	$8.47 \times 10^2 \pm 0.79^{d}$	0.5	0.09	0.5
DG(40:2)	$1.14 \times 10^4 \pm 0.40^{cd}$	$1.13 \times 10^4 \pm 0.22^{c}$	$0.46 \times 10^4 \pm 0.16^{d}$	0.9	0.2	0.08
eicosapentaenoic acid	$1.29 \times 10^4 \pm 0.32^{c}$	$1.03 \times 10^4 \pm 0.26^{cd}$	$0.46 \times 10^4 \pm 0.18^{d}$	0.9	0.07	0.1
3-carboxy-4-methyl-5-propyl-2-furanpropionic acid	$5.20 \times 10^5 \pm 1.10^{ac}$	$4.55 \times 10^5 \pm 1.21^{c}$	$1.84 \times 10^5 \pm 0.39^{bd}$	0.96	0.05	0.06
pimelic acid	$3.34 \times 10^4 \pm 0.75^{a}$	$1.61 \times 10^4 \pm 0.22^{ab}$	$0.76 \times 10^4 \pm 0.16^{b}$	0.2	0.01	0.2
palmitoleic acid	$2.04 \times 10^4 \pm 0.43^{c}$	$2.18 \times 10^4 \pm 0.27^{c}$	$3.01 \times 10^4 \pm 0.24^{d}$	0.99	0.09	0.08
eicosadienoic acid	$3.77 \times 10^3 \pm 0.45^{a}$	$4.81 \times 10^3 \pm 0.26^{ab}$	$4.89 \times 10^3 \pm 0.24^{b}$	0.1	0.05	0.8
PC(38:2)	$0.34 \times 10^7 \pm 0.15^{c}$	$2.26 \times 10^7 \pm 0.71^{d}$	$0.68 \times 10^7 \pm 0.07^{cd}$	0.06	0.9	0.1
PC(38:4)	$2.70 \times 10^5 \pm 0.82^{cd}$	$1.75 \times 10^5 \pm 0.76^{c}$	$4.34 \times 10^5 \pm 0.82^{d}$	0.4	0.5	0.06
PC(36:4)	$0.72 \times 10^4 \pm 0.10^{ac}$	$1.70 \times 10^4 \pm 0.25^{bd}$	$0.85 \times 10^4 \pm 0.11^{c}$	0.05	0.96	0.08
PC(36:5)	$3.85 \times 10^3 \pm 1.71^{c}$	$9.98 \times 10^3 \pm 1.80^{d}$	$9.65 \times 10^3 \pm 1.41^{d}$	0.06	0.06	0.99
PC(32:0)	$0.62 \times 10^5 \pm 0.24^{c}$	$2.13 \times 10^5 \pm 0.37^{d}$	$2.03 \times 10^5 \pm 0.40^{d}$	0.08	0.08	0.99
PC(44:7)	$1.59 \times 10^4 \pm 0.57^{a}$	$3.98 \times 10^4 \pm 0.59^{ab}$	$4.34 \times 10^4 \pm 0.66^{b}$	0.1	0.02	0.6
PC(40:1)	$0.54 \times 10^4 \pm 0.24^{a}$	$2.40 \times 10^4 \pm 0.68^{b}$	$1.08 \times 10^4 \pm 0.12^{ab}$	0.04	0.7	0.2
PC(34:3)	$1.16 \times 10^4 \pm 0.32^{c}$	$3.38 \times 10^4 \pm 0.78^{d}$	$1.69 \times 10^4 \pm 0.28^{cd}$	0.07	0.8	0.2

PC(38:5)	$0.83 \times 10^4 \pm 0.34^{c}$	$3.57 \times 10^4 \pm 1.07^{d}$	$1.12 \times 10^4 \pm 0.22^{cd}$	0.09	0.97	0.1
LPC(14:0)	$3.90 \times 10^5 \pm 0.47^{ab}$	$5.72 \times 10^5 \pm 0.87^{a}$	$3.70 \times 10^5 \pm 0.33^{b}$	0.1	0.9	0.05
LPC(17:0)	$0.17 \times 10^5 \pm 0.06^{c}$	$1.02 \times 10^5 \pm 0.35^{d}$	$0.37 \times 10^5 \pm 0.10^{cd}$	0.09	0.9	0.2
LPC(16:0)	$1.86 \times 10^5 \pm 0.04^{ab}$	$2.30 \times 10^5 \pm 0.32^{a}$	$1.46 \times 10^5 \pm 0.21^{b}$	0.3	0.4	0.02
LPC(22:0)	$0.47 \times 10^4 \pm 0.15^{ac}$	$1.07 \times 10^4 \pm 0.17^{d}$	$1.07 \times 10^4 \pm 0.14^{bd}$	0.06	0.03	0.9
PE(40:4)	$3.84 \times 10^5 \pm 1.01^{ab}$	$1.86 \times 10^5 \pm 0.81^{a}$	$6.00 \times 10^5 \pm 1.10^{b}$	0.2	0.3	0.008
PE(40:5)	$3.95 \times 10^5 \pm 0.84^{cd}$	$3.45 \times 10^5 \pm 0.45^{c}$	$5.69 \times 10^5 \pm 0.96^{d}$	0.7	0.4	0.09
PE(29:1)	$0.81 \times 10^4 \pm 0.18^{a}$	$1.46 \times 10^4 \pm 0.15^{b}$	$1.26 \times 10^4 \pm 0.17^{ab}$	0.05	0.2	0.8
PE(38:5)	$8.16 \times 10^3 \pm 0.44^{ac}$	$0.73 \times 10^3 \pm 0.36^{bd}$	$1.48 \times 10^3 \pm 0.28^{d}$	0.05	0.09	0.96
PE(38:3)	$3.02 \times 10^4 \pm 0.53^{a}$	$4.70 \times 10^4 \pm 1.13^{ab}$	$6.49 \times 10^4 \pm 0.71^{b}$	0.6	0.03	0.2
PE(40:9)	$0.70 \times 10^3 \pm 0.13^{c}$	$1.17 \times 10^3 \pm 0.27^{cd}$	$1.34 \times 10^3 \pm 0.17^{d}$	0.2	0.09	0.8
LPE(22:5)	$2.79 \times 10^3 \pm 0.63^{a}$	$1.15 \times 10^3 \pm 0.22^{b}$	$1.73 \times 10^3 \pm 0.35^{ab}$	0.02	0.1	0.6
LPE(16:0)	$0.88 \times 10^4 \pm 0.12^{c}$	$2.18 \times 10^4 \pm 0.57^{d}$	$1.00 \times 10^4 \pm 0.04^{c}$	0.06	0.97	0.09
LPE(22:0)	$5.69 \times 10^3 \pm 1.12^{c}$	$4.15 \times 10^3 \pm 0.85^{cd}$	$2.64 \times 10^3 \pm 0.44^{d}$	0.7	0.08	0.3
PG(31:2)	$0.68 \times 10^5 \pm 0.19^{ac}$	$1.44 \times 10^5 \pm 0.24^{bd}$	$1.46 \times 10^5 \pm 0.16^{d}$	0.05	0.08	0.98
LSM(18:0)	$1.02 \times 10^5 \pm 0.16^{a}$	$3.16 \times 10^5 \pm 0.19^{b}$	$1.43 \times 10^5 \pm 0.14^{a}$	0.003	0.96	0.004
SM(34:1)	$0.76 \times 10^5 \pm 0.15^{a}$	$2.09 \times 10^5 \pm 0.39^{b}$	$1.14 \times 10^5 \pm 0.07^{ab}$	0.03	0.7	0.2
SM(42:0)	$2.46 \times 10^3 \pm 0.21^{ab}$	$3.35 \times 10^3 \pm 0.35^{a}$	$2.02 \times 10^3 \pm 0.29^{b}$	0.3	0.4	0.03
PA(44:1)	$0.17 \times 10^7 \pm 0.07^{c}$	$1.01 \times 10^7 \pm 0.31^{d}$	$0.31 \times 10^7 \pm 0.03^{cd}$	0.06	0.9	0.1
PA(40:6)	$0.58 \times 10^5 \pm 0.18^{a}$	$1.21 \times 10^5 \pm 0.18^{b}$	$1.26 \times 10^5 \pm 0.14^{b}$	0.04	0.03	0.98
PA(39:0)	$1.01 \times 10^4 \pm 0.25^{a}$	$6.51 \times 10^4 \pm 2.07^{b}$	$3.58 \times 10^4 \pm 0.61^{ab}$	0.05	0.4	0.5
PA(42:4)	$1.50 \times 10^4 \pm 0.57^{cd}$	$1.50 \times 10^4 \pm 0.36^{c}$	$2.72 \times 10^4 \pm 0.45^{d}$	0.8	0.3	0.09
PI(34:1)	$3.11 \times 10^4 \pm 1.20^{a}$	$6.71 \times 10^4 \pm 0.94^{b}$	$7.25 \times 10^4 \pm 0.91^{b}$	0.05	0.02	0.8
PI(32:0)	$3.33 \times 10^4 \pm 0.73^{ac}$	$3.75 \times 10^4 \pm 0.36^{c}$	$4.89 \times 10^4 \pm 0.16^{bd}$	0.9	0.05	0.08
PI(36:1)	$2.76 \times 10^3 \pm 0.57^{c}$	$3.60 \times 10^3 \pm 0.51^{cd}$	$4.95 \times 10^3 \pm 0.67^{d}$	0.9	0.07	0.1
PS(36:1)	$1.55 \times 10^6 \pm 0.55^{c}$	$4.79 \times 10^6 \pm 0.96^{d}$	$2.45 \times 10^6 \pm 0.61^{cd}$	0.09	0.8	0.3
PS(38:5)	$2.21 \times 10^5 \pm 0.94^{ab}$	$1.94 \times 10^5 \pm 0.49^{a}$	$4.19 \times 10^5 \pm 0.66^{b}$	0.1	0.2	0.04

PS(42:4)	$2.68 \times 10^3 \pm 1.15^a$	$5.27 \times 10^3 \pm 0.89^{ab}$	$7.49 \times 10^3 \pm 1.22^b$	0.2	0.008	0.2
PS(32:3)	$5.36 \times 10^3 \pm 1.70^c$	$3.65 \times 10^3 \pm 1.23^{cd}$	$1.49 \times 10^3 \pm 0.37^d$	0.7	0.07	0.2
PS(38:4)	$1.57 \times 10^4 \pm 0.60^a$	$2.02 \times 10^4 \pm 0.43^a$	$3.52 \times 10^4 \pm 0.31^b$	0.99	0.04	0.03
PS(38:2)	$0.54 \times 10^4 \pm 0.16^c$	$1.59 \times 10^4 \pm 0.35^{bd}$	$0.46 \times 10^4 \pm 0.08^{ac}$	0.08	0.9	0.04
PS(42:8)	$0.76 \times 10^3 \pm 0.21^{ac}$	$2.49 \times 10^3 \pm 0.56^{bd}$	$1.05 \times 10^3 \pm 0.20^c$	0.02	0.8	0.07

Supplementary Table 5: Abundance of lipids in oocytes that differed between groups (NW; n=5, OB, n=7; OBD, n=6). Results are presented as mean ± SEM. Different superscripts within the same row indicate difference (^{ab}, $P < 0.05$) or tendency for difference (^{cd}, $P < 0.1$) between groups using one-way ANOVA with post-hoc Tukey's multiple comparison tests.

Lipid species	NW	OB	OBD	P value (NW x OB)	P value (NW x OBD)	P value (OB x OBD)
TG(50:1)	$4.95 \times 10^5 \pm 0.36^a$	$4.51 \times 10^5 \pm 0.35^{ab}$	$3.25 \times 10^5 \pm 0.59^b$	0.7	0.04	0.1
TG(50:1)	$1.66 \times 10^5 \pm 0.17^{cd}$	$1.71 \times 10^5 \pm 0.19^c$	$1.06 \times 10^5 \pm 0.23^d$	0.99	0.1	0.06
TG(54:1)	$4.73 \times 10^4 \pm 0.73^{cd}$	$6.07 \times 10^4 \pm 0.90^c$	$3.40 \times 10^4 \pm 0.69^d$	0.5	0.5	0.06
TG(58:9)	$1.28 \times 10^4 \pm 0.31^a$	$0.62 \times 10^4 \pm 0.10^b$	$0.57 \times 10^4 \pm 0.13^b$	0.05	0.04	0.96
TG(61:2)	$1.11 \times 10^4 \pm 0.13^c$	$1.86 \times 10^4 \pm 0.13^d$	$1.21 \times 10^4 \pm 0.30^{cd}$	0.09	0.9	0.1
TG(52:2)	$4.42 \times 10^3 \pm 0.39^a$	$3.29 \times 10^3 \pm 0.49^{ab}$	$2.33 \times 10^3 \pm 0.57^b$	0.6	0.05	0.2
TG(52:2)	$4.28 \times 10^4 \pm 0.10^a$	$3.37 \times 10^4 \pm 0.50^{ab}$	$2.43 \times 10^4 \pm 0.42^b$	0.3	0.01	0.2
TG(30:0)	$0.62 \times 10^4 \pm 0.08^{ac}$	$0.91 \times 10^4 \pm 0.08^c$	$1.80 \times 10^4 \pm 0.45^{bd}$	0.7	0.02	0.07
DG(24:0)	$3.31 \times 10^3 \pm 0.40^{ab}$	$2.80 \times 10^3 \pm 0.38^a$	$5.07 \times 10^3 \pm 0.57^b$	0.6	0.04	0.2
docosapentaenoic acid	$0.66 \times 10^4 \pm 0.15^c$	$1.37 \times 10^4 \pm 0.34^{cd}$	$1.87 \times 10^4 \pm 0.50^d$	0.3	0.09	0.6
octadecanedioic acid	$0.93 \times 10^2 \pm 0.15^{ac}$	$1.24 \times 10^2 \pm 0.16^c$	$1.60 \times 10^2 \pm 0.11^{bd}$	0.4	0.01	0.09
oleic acid	$0.77 \times 10^4 \pm 0.19^c$	$0.97 \times 10^4 \pm 0.10^{cd}$	$1.28 \times 10^4 \pm 0.12^d$	0.8	0.06	0.1
heptadecenoic acid	$1.88 \times 10^3 \pm 0.12^c$	$1.95 \times 10^3 \pm 0.17^c$	$2.40 \times 10^3 \pm 0.14^d$	0.9	0.06	0.08
stearidonic acid	$0.75 \times 10^4 \pm 0.11^{ac}$	$1.10 \times 10^4 \pm 0.16^c$	$1.83 \times 10^4 \pm 0.33^{bd}$	0.5	0.01	0.07
PC(46:5)	$2.82 \times 10^3 \pm 0.18^a$	$5.04 \times 10^3 \pm 0.57^{ab}$	$6.35 \times 10^3 \pm 1.18^b$	0.2	0.02	0.4
PE(42:2)	$6.65 \times 10^3 \pm 1.42^a$	$2.97 \times 10^3 \pm 0.44^b$	$4.77 \times 10^3 \pm 1.24^{ab}$	0.05	0.4	0.4

SM(16:1)	$1.87 \times 10^4 \pm 0.14^a$	$3.49 \times 10^4 \pm 0.64^{ab}$	$5.15 \times 10^4 \pm 1.29^b$	0.4	0.05	0.3
PA(40:3)	$0.22 \times 10^4 \pm 0.02^c$	$0.73 \times 10^4 \pm 0.25^{cd}$	$1.43 \times 10^4 \pm 0.53^d$	0.6	0.07	0.3
PS(34:0)	$2.86 \times 10^3 \pm 0.30^a$	$3.87 \times 10^3 \pm 0.56^a$	$6.72 \times 10^3 \pm 1.08^b$	0.7	0.007	0.02

Supplementary Table 6: Primer sequences of housekeeping gene and genes of interest.

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')	Product size (bp)	Gene Bank accession number
GAPDH	GATCCCGCCAACATCAAATG	ACATTGGGGCATCAGCAGAA	151	NM_001163856.1
CRAT	TCCCCAAGTCGGAGAAGCTG	CTGAGCGGATGGTGTCGGTA	150	XM_023629103.1
CPT1B	CTTCCGCCAAACCCTGAAAC	ACTAGGCACGGGAAGCTTGG	172	XM_023631561.1
PDK4	CAGTTGACCCAGTCACCAATCA	CTTGGACCACTGCTGACACG	180	XM_001493731.6
PDP1	CCCGGAATCCCAGTCAGAAG	GGGGATGAGCAGCAGAGATG	158	XM_023648692.1
LDHA	CACGTCAGCAGGAGGGAGAA	TAACGGAACCGGGCTGAATC	223	NM_001144880.1
FASN	GGAAGACACGGCAGCTCTGA	TCCAGGCTCTGCTCCCTTTC	201	XM_023651730.1
MTHFD2	AATGTGGACGGCCTCCTTGT	GGGTAGCCGGTAACATGGAAT	157	XM_005599899.3
SREBF1	GCTCCTTGCAAACCCAGGTC	ACTCGCTGCCTTCACAGTGG	206	XM_023653674.1
SREBF2	ATCCAGCAGCAGGTGCAGAC	TCTGGACTGCAGCCATGACA	217	XM_005606691.3
CCND2	CCAGCCGAATGACAAGTTGC	CTTGGCCAGCAGGTCTACCA	199	NM_001309189.1
LHCGR	CGGAGGAACCTCTCCCGACTAT	GGAGGTTGTCAAAGGCATTAGC	151	XM_023619111.1
STAR	AGGCCATGGGAGAGTGGAAC	ATGCCAGCCAACACACAGGT	181	NM_001081800.3
CYP11A1	ACCGCCTCCTAGCAAGCAAC	ACTTCCTCCCGCAGCATCTC	160	NM_001082521.1
CYP19A1	ATGGGCATGCATGAGAATGG	CAACGCATTGGTGACCTCGT	174	NM_001081805.3