Does exercise mitigate advanced maternal age's effects on pregnancy outcomes in a mouse model?

by

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Abstract

Advanced maternal age mothers are at greater risk of pregnancy complications such as preeclampsia and stillbirth. In humans, exercise benefits the mother and fetus, reducing the risk of developing adverse conditions. I aimed to evaluate the impact of exercise on fetal growth, survival and placental function in a mouse model. I studied three groups of female mice: young, aged, and aged exercise. I collected females at day 11 of gestation and assessed reproductive performance and placental transcriptome.

I observed a significant difference in maternal weight at the time of mating, however this reduction in weight of the aged females due to exercise did not improve pregnancy outcomes. Young females had more viable fetuses and fewer resorptions than aged, and aged exercise mothers. Fetuses from advanced maternal age mothers that exercised before and during pregnancy showed reduced fetal weight and size compared with those from young females, whereas fetuses of advanced maternal age mothers that did not exercise did not differ from those of young mothers. I observed no difference in the analysis of the differentially expressed genes, suggesting that the exercise treatment did not mitigate the effects of advanced maternal age.

Keywords: mouse, placenta, advanced maternal age, exercise

Dedication

To my mom and dad, thank you for all your support throughout my journey, for always encouraging me to do my best and take advantage of every opportunity.

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Chapter 1. Introduction

1.1. Advanced Maternal Age

Advanced maternal age (AMA) refers to pregnancy over 35 years of age. The number of advanced maternal age mothers is increasing due to socioeconomic and educational factors, including the increasing use and knowledge of birth control, delayed pregnancies due to lifestyle choices, subfertility and more developed assisted reproductive technologies, and multiparous women continuing childbearing (Haakstad 2019; Lean, Derricott, et al. 2017).

This steady increase in advanced maternal age can be seen especially in Western countries such as Canada, where the mean age of mothers at delivery was 29 years old in 2001 and increased to 31 years old in 2021. In Canada, 16% of live births were to mothers over 35 years old in 2000, rising to 18% in 2010, and to 26% in 2022 (Government of Canada 2023). In England and Wales, the rate of advanced maternal age mothers went from 49.7 live births per 1000 women in 2000 to 76.7 in 2021 (Office for National Statistics (ONS) 2023).

1.1.1. Aneuploidy

There is a well-known association between increasing maternal age and the frequency of aneuploidy, which is an incorrect number of chromosomes in a cell (Mikwar, MacFarlane, and Marchetti 2020). Chromosome anomalies lead to an increased risk of spontaneous miscarriage, being responsible for up to 50% of spontaneous miscarriages in women younger than 35 years and 75% in older mothers (McCallie et al. 2019). The most studied aneuploidies are the ones compatible with life, such as trisomy 21 (Down syndrome), trisomy 13 (Patau syndrome) and trisomy 18 (Edwards syndrome), with a prevalence per 10,000 births of 26.43, 2.79 and 7.42 respectively, in Europe 2020 (“European Platform on Rare Disease Registration,” n.d.; X.-H. Zhang et al. 2017).

Trisomies for all human chromosomes have been identified, and Ljunger et al. found that trisomies occurred in 60% of the anomalies identified in miscarriages. But

since this type of abnormality is usually strongly selected against in early pregnancy, they are rarely clinically recognized, even when the percentage of spontaneous abortions related to the chromosomal abnormalities is high, such as trisomy 16 and 22 (14% and 3.5% of the abortions) (Ljunger et al. 2005). Most monosomic embryos are spontaneously aborted in the early stages of development, leading to aneuploidy presence in 35% of spontaneous abortions (Mikwar, MacFarlane, and Marchetti 2020).

In 1981, Ernest and Hook found the rates of all aneuploidies rose from about 1 per 500 cases at maternal age < 20 to 1 per 180 cases at maternal age 35 and 1 per 20 cases at maternal age 45 (Hook 1981). More recently, in Korea, they found that the rate of Down syndrome and Edwards syndrome increases exponentially with age, where the odds ratio of all the fetal aneuploidies increases by 1.160 times per one-year increase in maternal age (Y. J. Kim et al. 2013). A study based in Zhejiang province, China, found similar results with rates of chromosomal abnormalities depending on maternal age, with 0.80 cases for maternal age < 20 and 24.99 for maternal age 35 - 47 per 10,000 births (X.-H. Zhang et al. 2017).

1.1.2. Other advanced maternal age complications

In addition to aneuploidies, advanced maternal age is associated with an increased risk of other pregnancy and childbirth complications, affecting both the mother's and the baby's health (T.-W. Kim, Park, and Park 2022). These risks include gestational diabetes (GD), fetal growth restriction (FGR), preeclampsia (PE), stillbirth and preterm birth (PTB) (Attali and Yogev 2021; Braggion et al. 2023; Lean, Derricott, et al. 2017; Napso et al. 2019; Tanaka et al. 2023). Advanced maternal age is related to a higher frequency of neonatal deaths, neonatal intensive care unit admissions, cervical incompetence, placental abruption, placenta previa and higher postpartum blood loss (Braggion et al. 2023; Lean, Derricott, et al. 2017; Tanaka et al. 2023). Risks of a hysterectomy, blood transfusion and cesarean delivery are also increased with advanced maternal age (Machado-Gédéon et al. 2023).

Lean et al. found that advanced maternal age has a population-attributable risk of 4.7% for stillbirths, based on the prevalence of advanced maternal age and the ratio of the risk of those exposed to advanced maternal age compared to the risk of those not exposed to the risk factor (the relative risk of stillbirth due to advanced maternal age)

(Lean, Derricott, et al. 2017). Advanced maternal age is an independent risk factor for poor pregnancy outcomes, with no consistent relationship between the number of pregnancies a woman has had (parity) and the effects of advanced maternal age (Lean, Derricott, et al. 2017). For example, nulliparous women aged ≥ 40 years have a higher risk of unscheduled cesarean delivery compared to nulliparous women < 40 (Braggion et al. 2023) .

Advanced maternal age as a risk factor is independent of maternal co-morbidities such as hypertension and diabetes. Interestingly, poor pregnancy outcomes in advanced maternal age persist despite factors that typically favour healthy pregnancy outcomes, such as higher socioeconomic status, pre-conceptional nutritional supplementation, non-smoking and high attendance to antenatal care (Lean, Derricott, et al. 2017).

Advancing age of over 44 years is associated with an increase in maternal mortality, as demonstrated by Tanaka et al. in a Japan study (Machado-Gédéon et al. 2023; Tanaka et al. 2023). They showed that the maternal mortality rate increased with advanced maternal age, with hemorrhagic stroke as the most common cause among pregnancies ≥ 40 years, with over half of these strokes being associated to preeclampsia (Tanaka et al. 2023). The physiological processes that relate maternal age to the health of the offspring might include deterioration of the reproductive system, such as decreased placenta and oocyte quality due to the age-dependent loss in efficiency of mitochondrial respiration. (T.-W. Kim, Park, and Park 2022; Mikwar, MacFarlane, and Marchetti 2020; Smits et al. 2023; Wilding 2014).

1.2. Decidualization, Implantation and Placentation

Decidualization is the process by which the endometrium undergoes structural and functional changes to support embryonic implantation, placentation, and pregnancy maintenance. In humans, decidualization is a process that is part of the menstrual cycle and initiates in the secretory phase of the cycle controlled by progesterone (Wang et al. 2020). The endometrium is a maternal uterine tissue essential during blastocyst implantation into the uterine wall. The endometrial stromal cells differentiate into larger and rounded decidual cells that are ready to support embryo implantation. If

implantation is successful, the differentiation of the stromal cells will spread further to form the maternal decidual compartment (Ramathal et al. 2010).

On the other hand, decidualization in mice is dependent on the attachment of the blastocyst. The preimplantation uterus is exposed to increased estrogen and progesterone to achieve receptivity. The blastocyst establishes initial communication at E4.0 between its mural trophectoderm and the maternal luminal epithelial cells (LE), positioning the inner cell mass (ICM) and polar trophectoderm towards the mesometrial (M) side (Panja and Paria 2021). The contact between the uterus and the blastocyst initiates decidualization through the action of estrogen and progesterone, transforming the stromal cells around the implanted embryo to undergo decidualization, forming the primary decidual zone (PDZ) (Hemberger, Hanna, and Dean 2020). Estrogen and progesterone are responsible for achieving the necessary endometrial thickness, which is a prerequisite for the implanting embryo (Pathare et al. 2023). During the next 3 days, decidual cells further differentiate and proliferate, forming the secondary decidual zone (SDZ), reaching full decidualization by E8 (Wang et al. 2020). To ensure direct interaction with the decidual cells, the luminal epithelial cells experience apoptosis under the influence of trophoblast cells in the site where the blastocyst makes contact (Panja and Paria 2021). This process is essential, and successful placentation requires proper communication between the trophoblast cells and the decidua (Hemberger, Hanna, and Dean 2020; Woods, Perez-Garcia, and Hemberger 2018).

In mice, trophoblast giant cells play a crucial role in facilitating the implantation and invasion of the conceptus into the uterus. While most trophoblast giant cells migrate only a limited distance into the decidua, a specific subset, the spiral artery-associated trophoblast giant cells, invades the spiral arteries that transport maternal blood to the implantation site (Cross 2005). In contrast, in humans the invasion into uterine tissues is initially made by the primary syncytial trophoblasts following implantation. These cytotrophoblast cells invade into the decidua as extravillous trophoblast (EVT) reaching as far as the inner third of the myometrium (Burton 2022; Turco and Moffett 2019).

The placenta has both maternal uterine and fetal components. The fetal portion of the placenta is derived from the blastocyst and made up of trophoblasts that form the majority of the placenta, while the maternal portion consists of the uterine tissue, particularly the endometrial tissue and the maternal blood vessels within the

endometrium that undergoes modifications to support the growing placenta. (Woods, Perez-Garcia, and Hemberger 2018). Positioned between the uterus and the fetus, the placenta plays a crucial role in pregnancy and in ensuring the fetus's well-being through several functions, including securing the conceptus to the uterine wall, producing hormones, creating a proper immune environment and transporting nutrients and oxygen, which requires remodelling the uteroplacental vasculature to increase blood flow (Wooldridge et al. 2022; Brislane et al. 2021; Panja and Paria 2021).

The mouse placenta comprises three structures: the maternal decidua, the junctional zone (JZ), and the labyrinth. The JZ secrets hormones and forms the middle layer of the placenta between the outermost giant cells and the innermost labyrinth and consists of spongiotrophoblasts and glycogen trophoblasts cells. The syncytiotrophoblast forms the nutrient transport surface within the labyrinth layer, and the labyrinth is characterized by intricate branching that facilitates the efficient exchange of oxygen, nutrients, and waste products by increasing the surface area between the maternal blood and the fetal capillaries, similar to the primary villi in the human placenta (Panja and Paria 2021; Cross 2005).

1.3. Decidualization, Implantation, and Placentation in advanced maternal age

Some advanced maternal age pregnancy complications are associated with the failure of correct placentation (Woods et al. 2017). Advanced maternal age-related placental dysfunction is a significant cause of fetal growth restriction (FGR), increasing the risk of stillbirth (identified in up to 65% of stillbirths), miscarriage, cerebral palsy and prematurity. However, placentation failure and FGR can even cause adverse outcomes in the progeny after many years, such as increased risk of cardiovascular diseases, diabetes mellitus, hyperinsulinemia and immune deficiencies (Woods, Perez-Garcia, and Hemberger 2018; Chae, Son, and Du 2022; Lean, Heazell, et al. 2017). One of the reasons for the increase in cesarean delivery rates in advanced maternal-age pregnancies is location anomalies of the placenta, such as placenta previa, present in 18.8% of women aged 35 or older (Serhat and Tugba 2021). In mice, defective decidualization by advanced maternal age affects trophoblast development, reduces the labyrinth size and increases the number of trophoblast giant cells, impacting the placental function and fetal growth.

Decidualization begins through the action of estrogen and progesterone during the secretory phase of the menstrual cycle in humans. As a result, the changes the endometrial lining undergoes are affected when the concentration of estrogen is abnormal in advanced age. Hormonal responsiveness and, therefore, levels of estrogen and progesterone decrease progressively with age, and around 50% of deregulated genes in the aged decidua are associated with the estrogen receptor-α (Esr1) or the Pgr binding site, which can further influence cellular differentiation during endometrial receptivity (Pathare et al. 2023; Woods et al. 2017). Advanced maternal age dysfunction in stromal cell proliferation can affect decidualization, endometrial receptivity, endometrial thickness, and embryo implantation. This is seen in in vitro mouse uterine stromal cells, where decidualization is delayed with downregulation of key regulatory genes like Prl8a2, Bmp2, Hand2, Hoxa10, Nr2f2, Igfbp5, Sfrp5, Ltf, Muc1 and Cdh1 in aged females (Pathare et al. 2023; Woods et al. 2017).

Sirtuin1 is a histone deacetylase that is known to regulate DNA repair, adipogenesis, oxidative stress, senescence and inflammation during aging. The expression of Sirtuin1 in mice is significantly decreased in the aged decidua, which is a critical age-related regulator of the Progesterone Receptor PGR actions for implantation. Sirtuin1 deficiency compromises stromal cell decidualization, leading to abnormal placentation and adverse pregnancy outcomes (Cummings et al. 2022). In female fetus placentas from advanced maternal age rats, there are changes in the expression of genes associated with the up-regulation of Igf2, suggesting beneficial changes related to nutrient transfer (Napso et al. 2019). Advanced maternal age reduces litter size, which cannot be fully explained by the increased resorption rate and may be attributed to implantation failure or peri-implantation loss. Even with the reduced litter size, fetuses are growth-restricted, possibly due to inadequate nutrient or oxygen delivery to the fetus (Care et al. 2015).

Carnitine palmitoytransferases (CPTs) catalyze the synthesis of acylcarnitines from fatty-acyl CoAs, which is essential to transport fatty acids into mitochondria for fatty acid oxidation. Mitochondrial oxidative damage in different tissues might result from decreased CPT1 expression with age. The conceptus, including the placenta, inherits maternal mitochondria, a major site where CPT1B is active. Low expression of CPT1B in the placenta, such as the one observed in advance maternal age, can lead to placental dysfunction due to the reduced ability of the placenta to regulate fatty acid

oxidation in response to metabolic challenges, including obesity which is common in advanced maternal age (Yong et al. 2023).

In the endometrium advanced maternal age can also alter the expression of genes associated with mitotic division, angiogenesis, immune response, and inflammation such as the upregulation of IL1A and IFNG, pro-inflammatory biomarkers in bovine cells in vitro, upregulation of the proinflammatory cytokine Il17rb and chemokines cxcl12 and cxcl14 in a murine model and downregulation of exosome-related genes essential for the signal exchange between the embryo and the endometrium to induce cell adhesion and migration (Pathare et al. 2023). Advanced maternal age was also shown to increase the endometrial inflammatory cell infiltration and levels of fibrotic changes in mares and aggravate the endometrial collagen deposition along with a decrease in collagen degradation, affecting functions such as decidualization by impairing the remodeling of stromal cells in rats (Pathare et al. 2023).

1.4. Exercise during Pregnancy

Exercise during pregnancy has many benefits for the mother and fetus. It is recommended for pregnant women to engage in at least 150 min of moderate-intensity physical activity per week (Silva et al. 2023). One benefit is the improvement of metabolic health in offspring. During the third trimester of pregnancy, there is also an inverse association between exercise and branched-chain amino acids, whose accumulation has been linked to obesity, type 2 diabetes, and aging (Silva et al. 2023).

Exercise also increases the expression of superoxide dismutase 3 (SOD3) in the placenta, which activates a pathway that changes the expression profile in the offspring's liver, regulating glucose homeostasis (Kusuyama et al. 2021). Engaging in exercise while pregnant can also lower the risk of gestational hypertension, and macrosomia all without increasing the risk of preterm birth, low birth weight, or growth restriction (Silva et al. 2023).

Differences in pregnancy outcomes have been identified depending on the level of exercise, with a 55% lower risk of developing gestational diabetes at higher pre-pregnancy exercise from a meta-analysis, or a four times higher risk of developing gestational diabetes at low total physical activity compared to those that had higher

levels of physical activity during early pregnancy from a case-control study with 200 patients (Tobias et al. 2011; Nasiri-Amiri et al. 2016). Finally, women can present a 40% reduction in the risk of developing preeclampsia when they engage in physical activity during pregnancy (Ferraro, Gaudet, and Adamo 2012; Jackson et al. 1995).

1.5. Placenta Changes with Exercise

Exercise regulates fetal growth, placental development and energy consumption by regulating mitochondrial adaptation in the placenta to support fetal development, and placental cytokine secretion, such as adiponectin, irisin and apelin. Placenta-secreted adiponectin reduces placental malfunction, increases cytotrophoblast differentiation, helps trophoblast invasion and improves insulin sensitivity (Chae, Son, and Du 2022). Increasing irisin improves glucose homeostasis, trophoblast differentiation and placenta development; low levels of irisin are found in preeclampsia and fetal growth abnormalities (Chae, Son, and Du 2022; Szumilewicz et al. 2017). An increase in placental apelin secretion regulates placental and fetal growth, improves vascularization and improves nutrient transport, such as placental trophoblast nutrient uptake, likely due to the upregulation of glucose transporters 1 and 3 (GLUT1 and GLUT3) (Chae, Son, and Du 2022; Pahlavani et al. 2023).

Exercise also induces changes in the expression of genes related to placental growth in mice such as insulin growth factor 1 (IGF-1), fibroblast growth factor 2 (FGF2), neurotrophin-4 (NT-4), and placental growth factor (PGF) (Kusuyama et al. 2020) . Exercise during pregnancy can also reverse the effects of maternal high-fat diet on the expression of genes associated with placental vascularization. These genes include apelin, vascular endothelial growth factor (VEGF), VEGF receptor 1 and hypoxia-inducible factor 1-α (HIF1-α) (Kusuyama et al. 2020). Exercise increases Apelin (APLN) levels in the maternal circulation and placental APLN, which enhances brown adipogenesis in the offspring through DNA demethylation at the promoter of the PR domain containing 16 (Prdm16) (Kusuyama et al. 2020).

Among the critical substrates transported through the placenta are lipids and fatty acids, which can be regulated with exercise during pregnancy in maternal obesity, shown by higher concentrations in the maternal circulation of beneficial fatty acids such as linoleic acid and omega 6 fatty acids as well as lower concentrations of saturated

fatty acids. Circulating levels of fatty acids are important since essential fetal fatty acids must be obtained from mothers and are necessary for fetal development (Silva et al. 2023; Ruebel et al. 2023; Mills et al. 2019). Differences due to maternal exercise are sometimes sex-dependent. For example, lipid and steroid metabolism are affected in males, such as the inhibition of the steroid hormone receptors ESR1 and PGR that have an essential role in placental development and energy metabolism, while muscle growth, vascular development, and growth factors are affected in females (Ruebel et al. 2023).

Other exercise-induced adaptations that increase oxygen and nutrient transfer delivery in the placenta are increased capillary volume, vasodilation and increased placental function capacity, including an increase in surface area and enhanced perfusion balance by improvement of blood flow and gas exchange (Brislane et al. 2021; Ferraro, Gaudet, and Adamo 2012).

Changes in placenta morphology associated with exercise during pregnancy include an increase in placental growth rate and gross volume as well as mitigation of changes caused by maternal high-fat diet such as decreased labyrinth thickness and increased decidua and junctional zone thickness (Kusuyama et al. 2020; Clapp et al. 2000).

1.6. Effects of Exercise on Long-Term Offspring Outcomes

FATP4, an acyl-CoA synthetase that activates long-chain fatty acids, is more expressed in the term placenta from physically active women, along with higher expression of SNAT2, a key amino acid transporter, compared to non-active women (Brett et al. 2015; Kusuyama 2021; Hutchinson et al. 2020). Placental FATP4 increased expression in active women (150 min of weekly moderate-intensity physical activity) indicates that exercise promotes fatty acid metabolism instead of storage, leading the fetuses to have lower adiposity (Hutchinson et al. 2020). This reflects the importance of nutrient transporters for glucose, amino acids, and fatty acids in placental function and pregnancy outcomes, particularly in the presence of pathologies such as FGR, preeclampsia (PE) and Gestational Diabetes Mellitus (GDM) (Hutchinson et al. 2020).

Studies in rodents from weaning to adulthood have demonstrated that maternal exercise can lead to either a decrease or no change in offspring's adult body weight (Kusuyama et al. 2020). Additionally, maternal exercise in Sprague-Dawley rats has been found to result in lower fat mass in male adult offspring but not in female adult offspring (Hutchinson et al. 2020). Exercise during pregnancies also gives rise to long-term vascular programming and benefits offspring´s heart rate according to an observational pilot study including 61 pregnant women (Brislane et al. 2021; Ferraro, Gaudet, and Adamo 2012; May et al. 2010).

1.7. Animal Models of advanced maternal age

Animal models are frequently used for studies of advanced maternal age and placental dysfunction in adverse pregnancy outcomes (Kusuyama et al. 2020; Lean, Derricott, et al. 2017). A systematic review of murine models of advanced maternal age found that advanced maternal age murine models range from 26 to 43 weeks old, but the early stages of reproductive decline in female murine occur from approximately 30-34 weeks of age (Dalton-O'Reilly et al. 2023). Most studies, therefore, use rats above 39 weeks old (~35 years in humans) and above 34 weeks old for mice to reflect the onset of reproductive aging in middle-aged females (Velazquez et al. 2016; Wooldridge et al. 2022). The young adult group should be from 13 to 26 weeks old (Flurkey, M. Currer, and Harrison 2007).

Many similarities have been identified in the placental phenotype of advanced maternal age in the mouse model and human pregnancies, particularly increased placental weight, decreased placental efficiency and altered uteroplacental vascular function (Lean, Derricott, et al. 2017). Reduced fetal weight in both advanced maternal age mice and women has been shown to increase rates of FGR (Dalton-O'Reilly et al. 2023). Lean et al. found a reduction in fetal: placental weight ratio in a mouse model (37.4% in advanced maternal age compared to controls), reflecting the severity of the phenotype (Lean, Heazell, et al. 2017).

In mice, the negative impact of advanced maternal age on reproductive outcomes includes a reduced number of total and viable pup, twice as many embryo resorptions (loss and absorption of embryonic tissue) and more non-viable pups, affecting 31% of advanced maternal age litters (Lean, Heazell, et al. 2017). Although

humans usually produce a single offspring at a time, litter size in murine is a good measure for fecundity, and therefore, reduced litter size and increased number of resorptions observed in advanced maternal age murine reproduces a similar phenotype to human pregnancies of advanced maternal age whereby the rate of miscarriage and stillbirth is increased (Dalton-O'Reilly et al. 2023).

1.8. Animal Models of Exercise

Voluntary wheel running (VWR) can be used to study exercise in the mouse model. Mice run spontaneously when they have access to running wheels, and in laboratory settings, mice have high activity levels and start running immediately after the dark cycle begins (Manzanares, Brito-da-Silva, and Gandra 2018). Running activities can vary from week to week in each individual mouse, a variation that is more pronounced in females and more significant in early adulthood (Bartling et al. 2017). Aging is an independent factor for total wheel activity, decreasing velocity and increasing break time (Bartling et al. 2017).

Novel environments induce stress, including the addition of wheels into cages. C57BL/6J mice respond to novel cage environments by increased wheel activity. Even then, voluntary wheel running gives access to exercise under non-stress conditions and does not disrupt the animal's natural nocturnal-diurnal rhythm (McMullan et al. 2016).

Mice that exercise have shown a 16% decrease in body mass, a 50% decrease in body fat, and an increase of 15% in lean mass compared to mice not exposed to running wheels. Even though exercise declines with age, exercising in running wheels prevents age-related changes in body mass and composition, reducing body fat and increasing the lean mass (McMullan et al. 2016).

1.9. Objectives

In this thesis, I evaluate the impact of exercise on fetal growth and placental function in a mouse model of advanced maternal age. More specifically, this thesis aims to (1) determine if exercise is associated with increased reproductive performance, e.g., the number of conceptuses, unviable fetuses, and fetal weight; and (2) identify genes

whose placental expression changes with exercise in advanced maternal age to assess the mechanisms by which exercise may act.

Chapter 2. Methods

2.1. Animal models

I studied three groups of female mice: Young, Aged, and Aged/Exercised. C57BL/6J mice were purchased from the Jackson Laboratory at 5 weeks (Young) or 30 weeks (Aged). I purchased 35 - 40 mice per group, anticipating that only half would get pregnant. I used separately ventilated cages with a 12:12 hour light:dark cycle. I provided the Aged/Exercised mice group with wireless running wheels (ENV-044 Mouse Low-Profile Wireless Running Wheel, Med Associates Inc) four weeks before mating. Wheels were randomized among the cages, and the exercise was quantified by remotely monitoring the running wheels throughout the experiment. Females in all groups were housed in cages of 4 but individually for one night at a time, on days 7, 14 and 21 after the wheel was added, to obtain individual measurements of physical activity in females with running wheels. Females in the Aged and Young groups were also single-housed on the same nights to control for the stress of single-housing. A fourth measurement was taken on the night before culling when they were single-housed after determining they were pregnant based on the increase in weight at GD10.5 (described below). Female mice were fed a breeder diet (breeding diet: Prolab RMH 3000, LabDiet, St. Louis, MO).

2.2. Mating

I paired all females with a Young male without a running wheel to control paternal effects across treatments. I paired a maximum of 3 females in a cage with a male for one night and mated 20~30 females weekly to get approximately 6 pregnancies/dissections a week. After mating I housed the females back in the same cages of four and they stayed there until pregnancy assessment. Pregnancy was assessed by weight gain 10 days later, based on a weight gain above 2.5 g. If pregnant, I single-housed pregnant females overnight, allowing us to get another individual measurement of exercise. I collected females at day 11.5 of gestation (where the day after mating was day 0.5). To avoid collecting females that were not pregnant, if the female had gained between 1.7 g and 2.5 g, the female would be weighed again on day 11 to see if the weight gain continued or reversed. If the female continued to gain

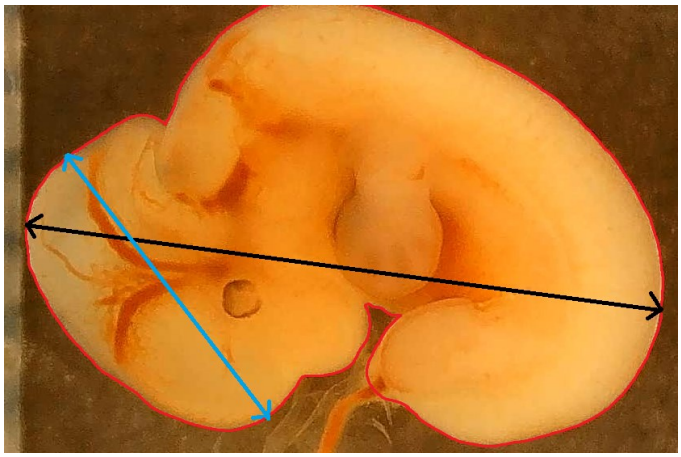
weight, the female would be collected. This technique led to only 5/66 mistakes in the collection of pregnant females who had only gained weight but were not pregnant. I never missed the collection of pregnant females.

2.3. Dissections

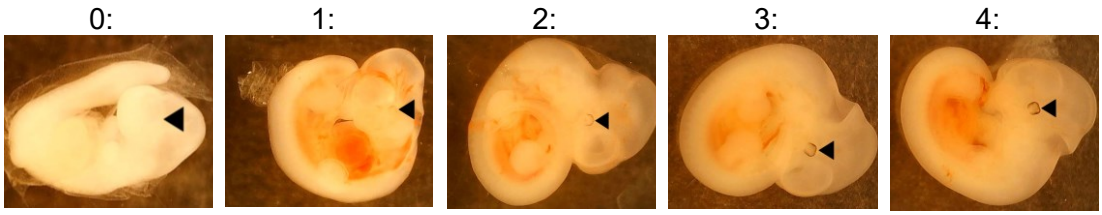
The uterus was dissected out and placed on ice at the time of collection. I also took a sample of maternal blood by cardiac puncture. From the uterus, I dissected on ice 2 typical-looking placentas (bigger size and not dark looking to avoid resorptions), snap-froze them on liquid nitrogen and stored them on dry ice until stored at -80 °C for RNA extraction. To reduce bias, the researchers dissecting the placentas were blinded to the age group of the mice. Fetuses from dissected placentas and the rest of the uterus were fixed in 4% paraformaldehyde (PFA) and stored at 4 °C. Maternal blood was centrifuged to obtain plasma and then stored at -20 °C.

After fixing the remainder of the uterus for 2 days, I dissected the rest of the placentas and fetuses in phosphate-buffered saline (PBS). I assessed fetal and placental weight, the number of viable and unviable conceptuses (resorptions and fetuses with weight below the cutoff of 0.0115 g, See Section V.III Viable Fetuses in the Results), and took an image of each fetus. The image was used to assess the cross-sectional area of the fetus in profile, eye spot pigmentation, the presence of digits, the head's width, and crown-rump length, which was performed blind to female treatment (Fig. 1). I collected fetal tails to obtain DNA to determine sex by PCR, enabling us to test whether one sex is more affected than the other. The rest of the fetus and the tail were stored in a small tube each in the -20 °C freezer. The placentas were stored in 70% ethanol tubes at 4 °C.

A) Crown-rump (C-R) Length (black), Head's width (blue) & Cross-sectional profile area (red):



B) Eye spot pigmentation:



C) Presence of digits:

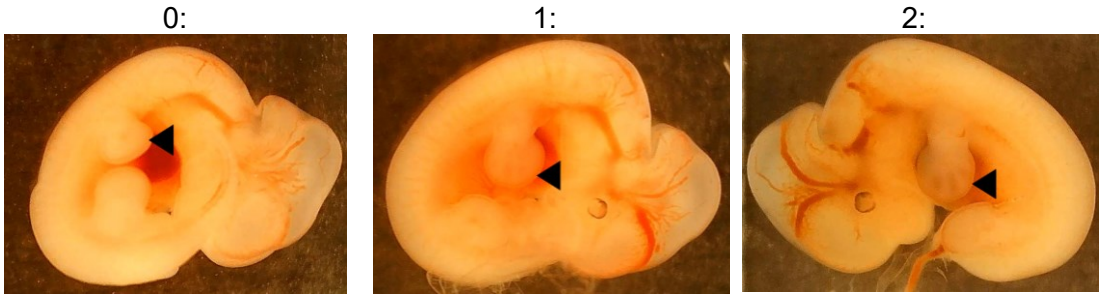


Figure 1. Measurements of fetus A) crown-rump (C-R) length (black), head's width (blue) & cross-sectional profile area (red), B) 5 levels of eye spot pigmentation and C) 3 levels of presence of digits. Black arrows indicate the area of the assessment.

2.4. PCR

DNA was extracted from the fetal tails with 60 µL lysate extraction buffer and 1 µL proteinase K (conc ≥10 mg/mL) by heating at 55 °C for 5 hours with vortexing at 2, 4 and 5 hours, followed by heat-inactivation of the proteinase K at 95 °C for 10 min and centrifugation at 13 000 for 15 min. 55 µL of the supernatant were added to 1 mL of

water and stored at 4 °C. The primers used to determine the genetic sex of the fetuses by detecting the X/Y chromosome are SX_F,5´GATGATTTGAGTGGAAATGTGAGGTA-3´; SX_R,5´CTTATGTTTATAGGCAT GCACCATGTA-3´ (McFarlane et al. 2013). PCR used a final volume of 20 µl with 40 cycles (denaturing at 95 °C, annealing at 50 °C and extension at 72 °C). PCR products were electrophoresed alongside the GeneRuler 100 bp Plus ladder from Thermo Scientific on 1.5% agarose gels and visualized with SYBR Safe under UV illumination.

2.5. Statistical analysis

Statistical analysis was performed in R. One-way Analysis of variance (ANOVA) was used to determine differences between groups in weight at mating and weight gain during pregnancy (weight gain = weight after dissection without uteri - weight at mating) followed by post-hoc Tukey test to determine specific differences between group means. To distinguish between viable and unviable fetuses, I used the fetal weight data from the Young group to determine the mean minus 3 standard deviations (SD), which I used as a cut-off for assessing fetal viability in the Aged groups. Those under the weight cutoff were considered unviable and these conceptuses were classified as resorptions.

I used Kendall´s correlation to determine the association between the average revolution per night among the Aged/Exercised mice with weight at mating and weight gain during pregnancy. To test if there is a correlation between the average activity during the three individual counts before mating and pregnancy outcomes, I performed Pearson correlation tests for litter size, weight at mating and average fetal weight and Kendall's rank correlation test for the number of resorptions and viable fetuses since these were not normally distributed.

I used ANOVA followed by Tukey test to test differences between groups in the number of viable fetuses, the number of unviable fetuses, the average presence of digits, and average fetal weights. Welch´s ANOVA is more robust to violations homogeneity of variances. Welch’s ANOVA followed by post-hoc test with Bonferroni correction were used to test differences between groups in average crown-rump length, head width, the levels of eyespot pigmentation and the cross-sectional area of the fetus in profile. To determine group differences in total number of conceptuses I used Kruskal-

Wallis test and multiple comparisons with Dunn's test since the data was not was not normally distributed.

Since the genotyping was unsuccessful for some of the fetuses, I performed analyses with and without sex. I used ANOVA followed by Tukey test to test differences between groups in sex ratio. I used two-way ANOVA to examine the effects of treatment and sex and the interaction between treatment and sex for crown–rump length, head width, fetal weight and the cross-sectional area of the body. I used the average of each parameter for each sex in all the females with at least 1 genotyped fetus.

I used logistic regression to assess the difference in the probability of getting pregnant between the groups. I used "Group" as the categorical independent variable and "Pregnant" as the dependent binary variable. Using logistic regression allows us to account for the repeated measurements, as some mice had multiple mating attempts.

2.6. RNA extraction

Total RNA was prepared using RNeasy Plus Mini Kit (Qiagen), including gDNA Eliminator columns to remove genomic DNA. The samples were analyzed in the Nanodrop for purity before sequencing. The samples were sent to UBC's Biomedical Research Centre Sequencing Core (BRC-Seq) for sequencing. Poly(A) enrichment was performed using magnetic oligo(dT) beads and library construction was performed using the standard protocol for the Illumina Stranded mRNA Prep Kit. Sequencing was performed on the Illumina NextSeq2000 with Paired End 59bp × 59bp reads. Read sequences were then aligned to the Mus Musculous (mm10) reference sequence using DRAGEN RNA app on Basespace Sequence Hub. Normalization was performed using DESeq2.

2.7. Differential expression analysis

I assessed placental transcriptomes using DESeq2 and calculated differential expression with a p-adjusted threshold of 0.05; p-adjusted is corrected for multiple testing using the Benjamini and Hochberg method as default in DESeq2. I used a multi-factor design to assess the interaction between groups and fetal sex in DESeq2 and used contrasts to obtain differentially expressed genes between each group pair (i.e.

Aged vs Young, Aged vs Aged/Exercised, Young vs Aged/Exercised). Heatmaps were made in R using Heatmap.2 using only genes with log fold change > 2. Gene enrichment of differentially expressed genes was performed using enrichGO in R. The GD9.5 and GD12.5 placenta single nuclei RNAseq data published by Marsh and Blelloch (https://figshare.com/projects/Single_nuclei_RNAseq_of_mouse_placental_labyrinth_development/92354) were used to estimate cell proportions using BisqueRNA. BisqueRNA is a bioinformatic method that estimates cell composition from bulk expression data using single-cell data as a reference. Significant differences were detected with ANOVA analysis in R for each cell type between Aged, Aged/Exercised, Young, and GD10.5 Young deconvoluted cell type proportions.

Chapter 3. Results

3.1. Level of voluntary locomotor activity among Aged Exercise mice

Some mice were consistently more active than others across the three nights of single-housed measurements; there was significant variation among females in the average revolutions during the 12 dark hours (p-value < 0.001; Fig. 2). In no case did the counts per minute stop abruptly during the night, confirming that none of the wheels stopped working during measurement. The average maximum resting time (continuous 0 counts, representing no use of the wheel) from the three counts before mating was 1.26 hours, with the minimum being 0.10 hours and the maximum being 3.05 hours.

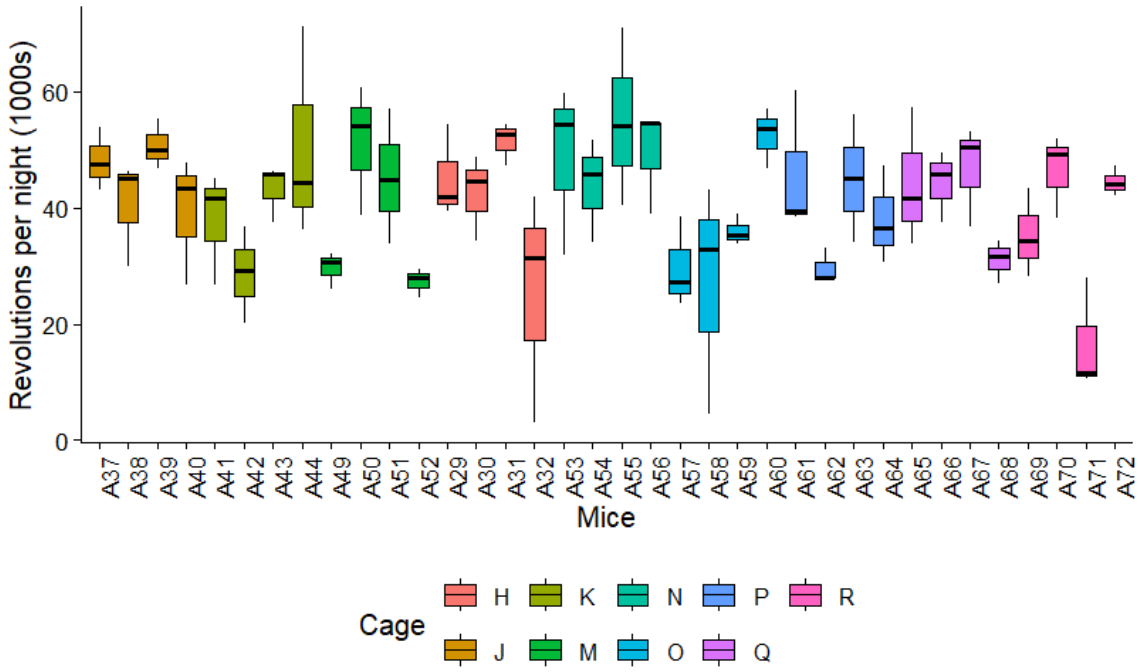


Figure 2. **Variation in the average revolutions per night per mouse. Each mouse was measured during the 12 dark hours on three nights. Mice were single-housed during measurement but were otherwise housed in cages of four, as shown by the colours.**

Of the 19 mice that became pregnant, I obtained an additional activity measurement for 16 while single-housing them the night before collection (GD10). Figure 3 shows that 10 out of the 16 measurements during pregnancy were < 20 revolutions/night (1000s) and 15 of the 16 mice showed lower activity during pregnancy than the average of their 3 pre-mating measurements.

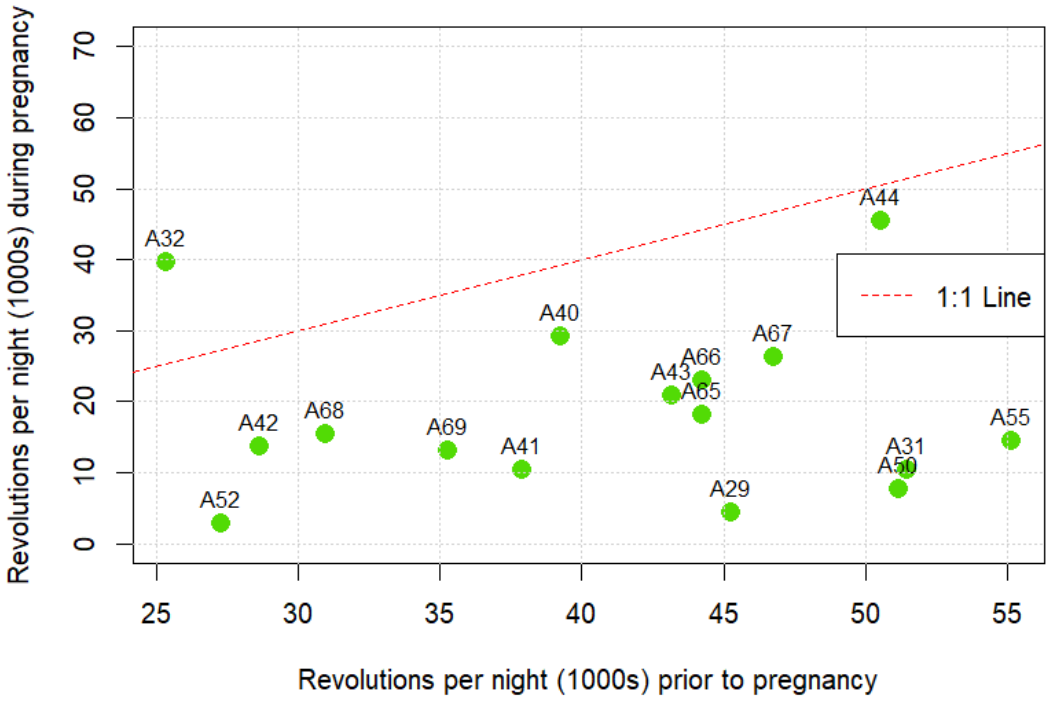


Figure 3. **Variation in the wheel revolutions per night per mouse at GD10.5 during pregnancy compared to the average of the three pre-pregnancy measurements.**

The average of the three counts before mating did not differ between mice that did or did not get pregnant (did get pregnant: 41.6 ± 2 revolutions per night (1000s); did not get pregnant: 39.78± 2.5 revolutions per night (1000s); t-test p-value = 0.57; Fig. 4).

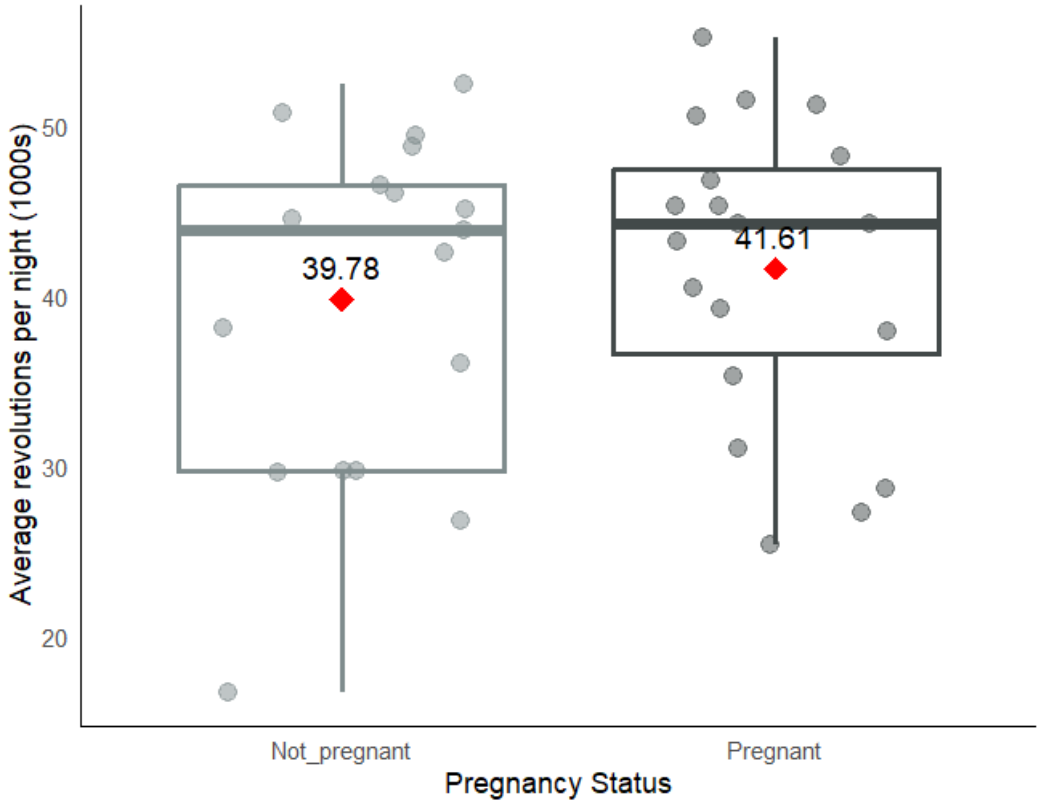


Figure 4. **Average wheel revolutions per mouse per night for the three measurements before mating in mice that became pregnant and those that did not, with the mean shown by the red diamonds and the median as the horizontal lines inside each boxplot.**

3.2. Probability of getting pregnant

Logistic regression showed no significant difference between Young, Aged and Aged Exercise females in the probability of pregnancy at each mating attempt (p-value > 0.05). Similarly, no difference in the number of mating attempts among mice who got pregnant was found (p-value > 0.05; Fig. 5)

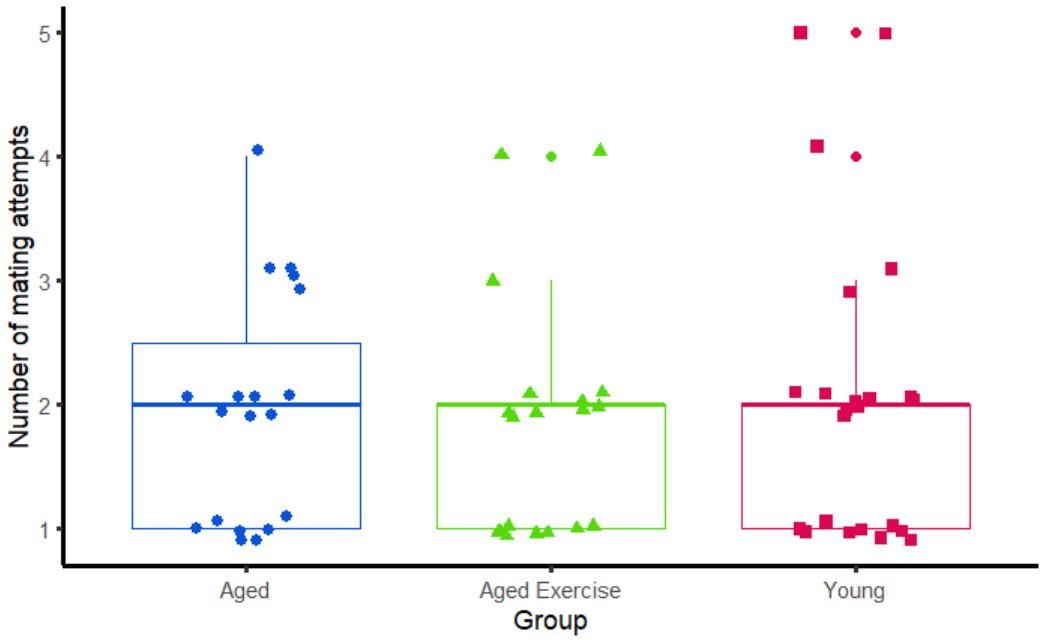


Figure 5. Number of mating attempts to become pregnant in each group.

3.3. Effect of activity on weight at mating

To assess the weight difference between the groups before their pregnancies I studied the weight at the mating day. Females in the Aged group were significantly heavier, at the time of the mating leading to a successful pregnancy, than females in the Aged/Exercised group, who were significantly heavier than the Young group (p-value < 0.0001; Fig. 6). Weight at mating showed no correlation within groups with the number of viable fetuses (p-value > 0.05; Fig. 7).

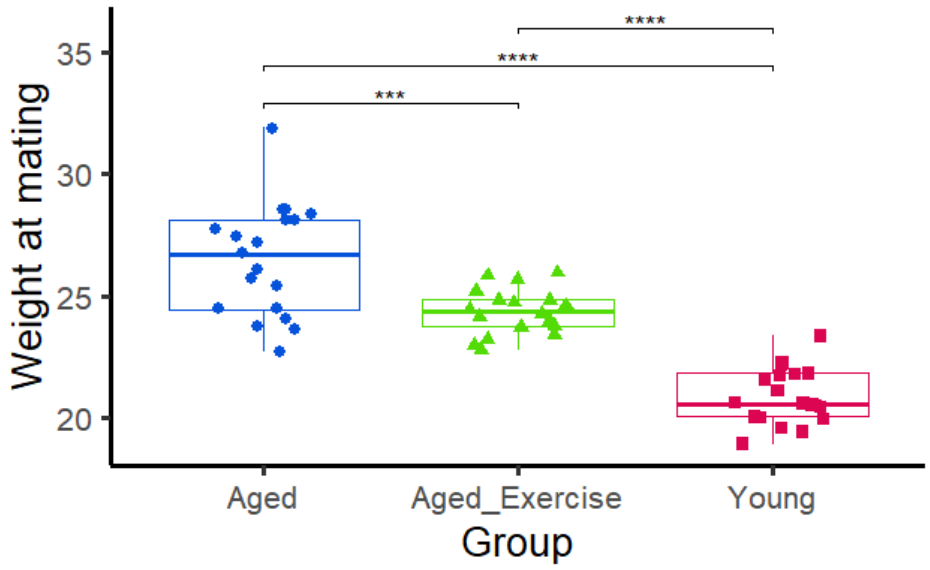


Figure 6. **Weight at the time of mating leading to successful pregnancy for the Aged, Aged/Exercised and Young groups. There is a significant overall difference among groups, and pairwise differences identified by the Tukey test are shown. p < 0.001: ***, p < 0.0001: ****.**

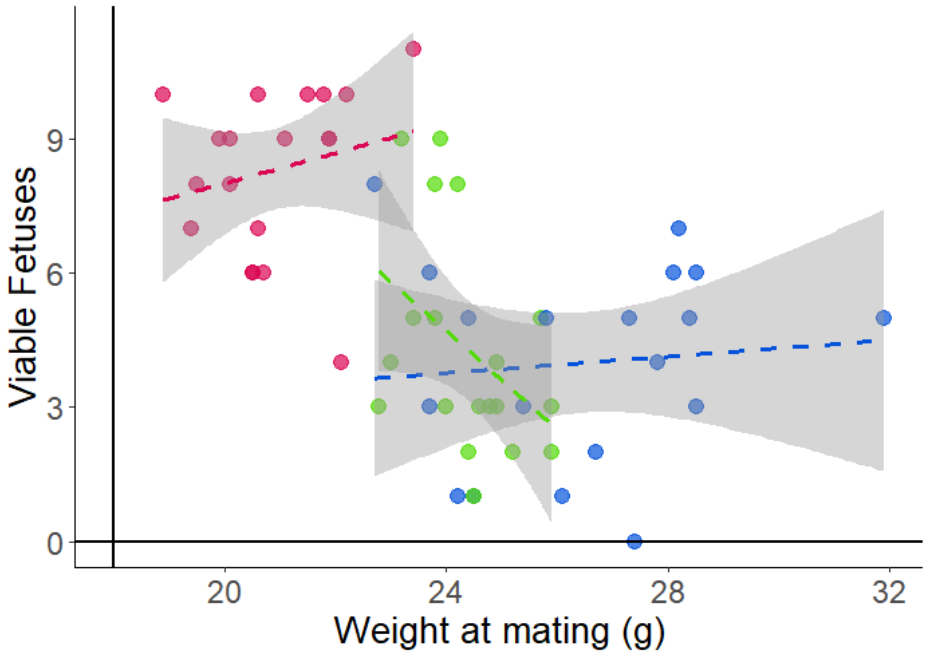


Figure 7. **Weight at the time of mating leading to successful pregnancy for the Aged, Aged/Exercised and Young groups. There is no correlation between any of the group's viable fetuses and weight at mating, as shown in Kendall's correlation for Aged: p > 0.1 & tau = 0.08, Aged/Exercised: p > 0.05 & tau = -0.32 and Young: p > 0.1 & tau = 0.19.**

3.4. Correlation of Activity and Pregnancy Measurements

To test if there is a correlation between the average activity during the three individual counts before mating and pregnancy outcomes, I performed Pearson correlation tests for litter size (total number of conceptuses), weight at mating and average fetal weight (Fig. 8). I performed Kendall's rank correlation test for the number of resorptions and viable fetuses since these were not normally distributed. Only the number of viable fetuses was correlated with average activity prior to pregnancy (tau = -0.51; p-value < 0.05; Fig. 8D), showing an inverse relationship.

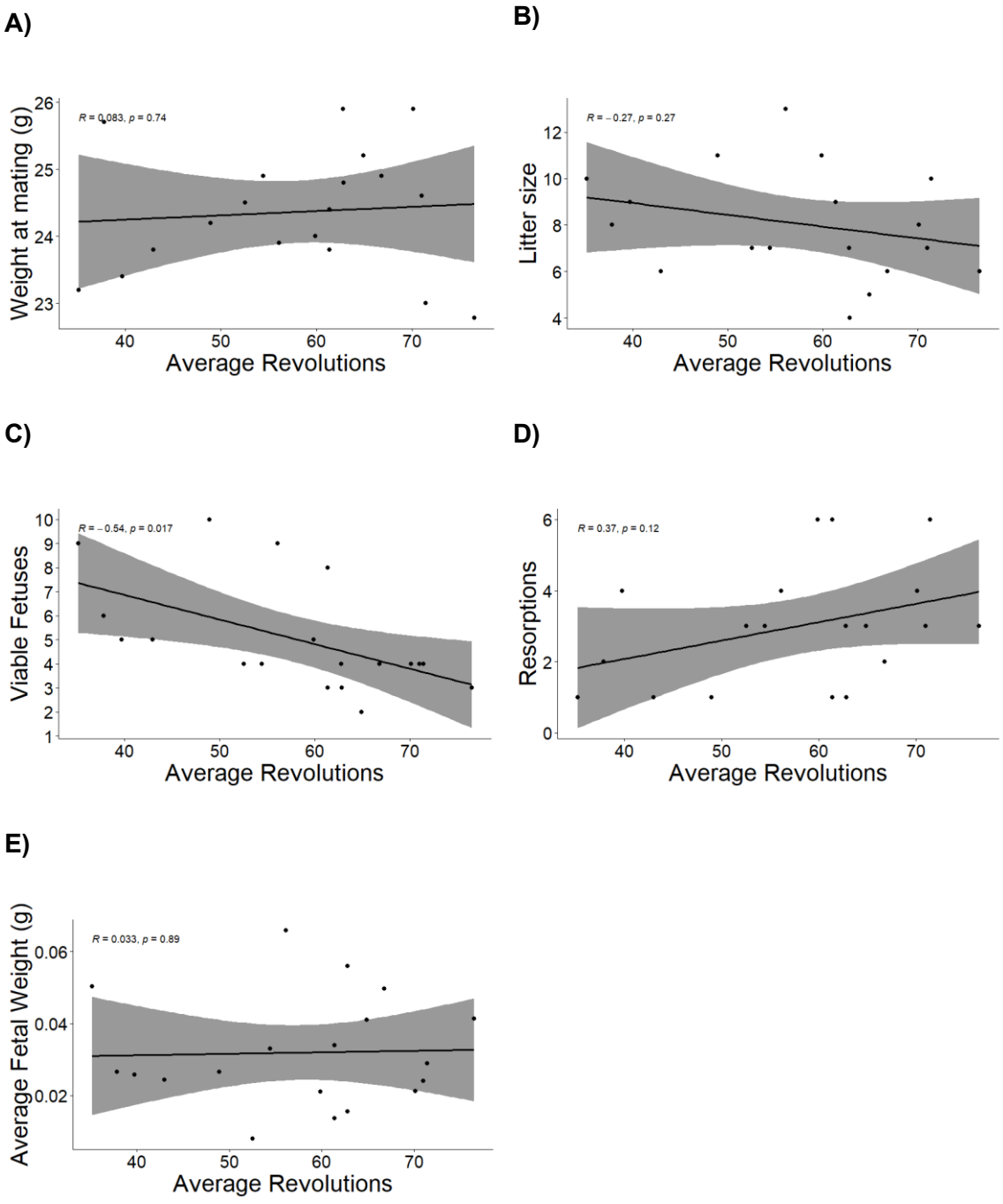


Figure 8. **Correlation between the average revolutions/night from the three counts before mating with A) Weight at mating, B) Litter size, C) Number of viable fetuses, D) Number of resorptions and E) Average fetal weight.**

3.5. Effect of Age and Activity on Pregnancy Outcomes

3.5.1. Weight

There were differences between groups in weight gain between the time of mating and dissection without uteri (i.e., changes in the female's mass that did not include the conceptuses), controlling for the number of viable fetuses. Weight gain was significantly lower in the Aged/Exercised group compared to the Aged group (p-value < 0.0001; Figure 9). There was no correlation between weight gain and the number of viable fetuses in any of the groups (p-value > 0.05; Figure 10).

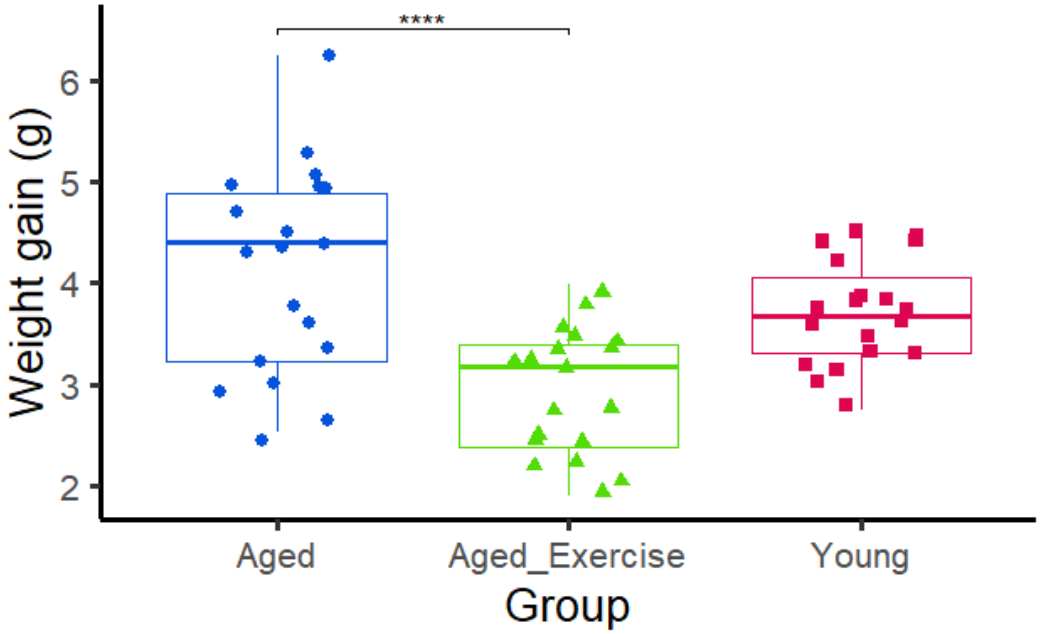


Figure 9. **Weight gain between the time of mating and dissection without uteri for the Aged, Aged/Exercised and Young groups. There is a significant difference between the Aged and Aged/Exercised groups, controlling for the number of viable fetuses (p-value < 0.0001: ****).**

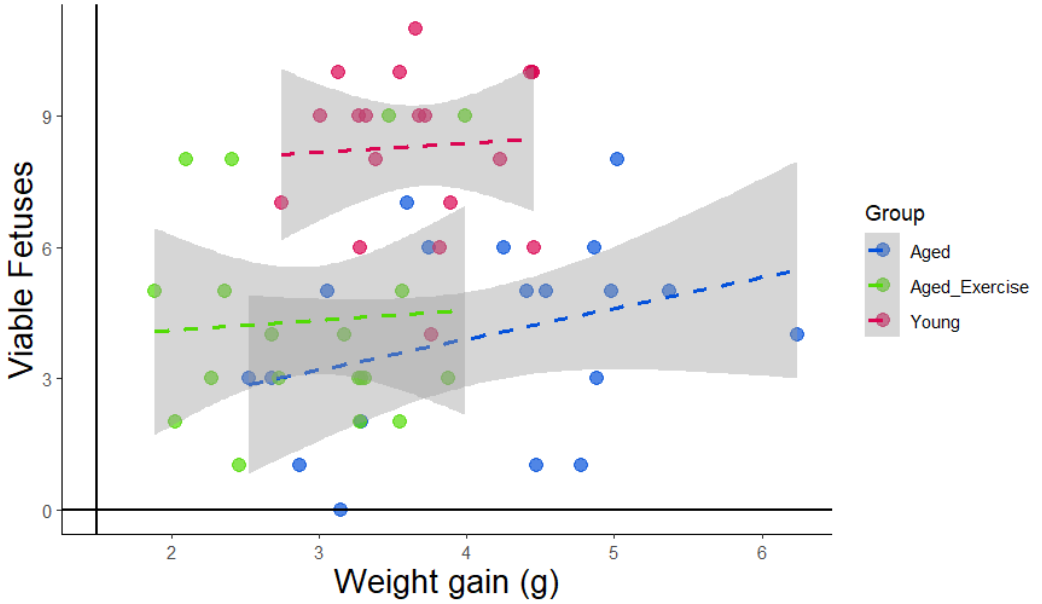


Figure 10. **Plot showing the correlation between weight gain and viable fetuses for the Aged, Aged/Exercised and Young groups. Kendall's correlation p-value > 0.05 in all groups with the correlation coefficient tau = Aged: 0.19, Aged/Exercised: 0.006 and Young: 0.013.**

3.5.2. Total Number of fetuses

Litter size (total number of conceptuses, including viable and unviable fetuses) was not normally distributed (Shapiro test p = 0.026) and was analyzed by a Kruskal-Wallis test. There was significant variation between the groups (p-value = 0.033; Fig. 11), and multiple comparisons with Dunn's test showed that Aged/Exercised mice had 1.42 fewer conceptuses than the Young mice (p-value = 0.042).

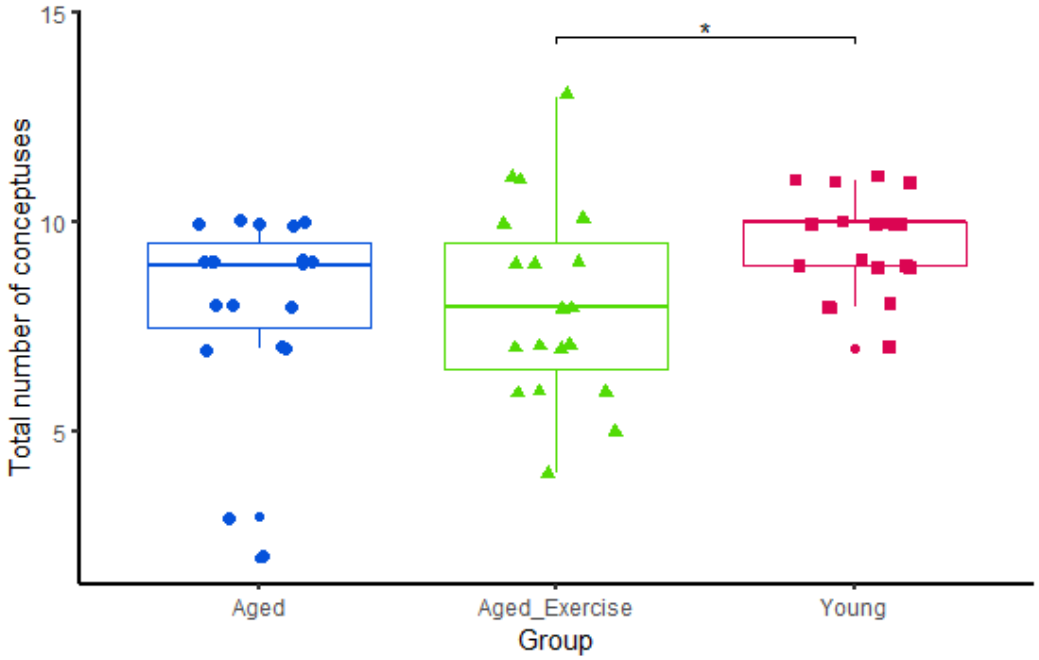


Figure 11. The total number of conceptuses of the Aged, Aged/Exercised and Young groups (Kruskal-Wallis $p < 0.05$: *).

3.5.3. Viable Fetuses

As described in the methods, I used a cut-off of the mean minus 3 SD from the Young fetal weight data (0.0115 g) to distinguish between viable and unviable fetuses in the three groups. With these limits, 18 of 94 fetuses from the Aged group, 13 of 95 from the Aged/Exercised, and 1 of 159 from the Young group were selected as unviable fetuses, not including conceptuses previously identified as resorptions during dissections.

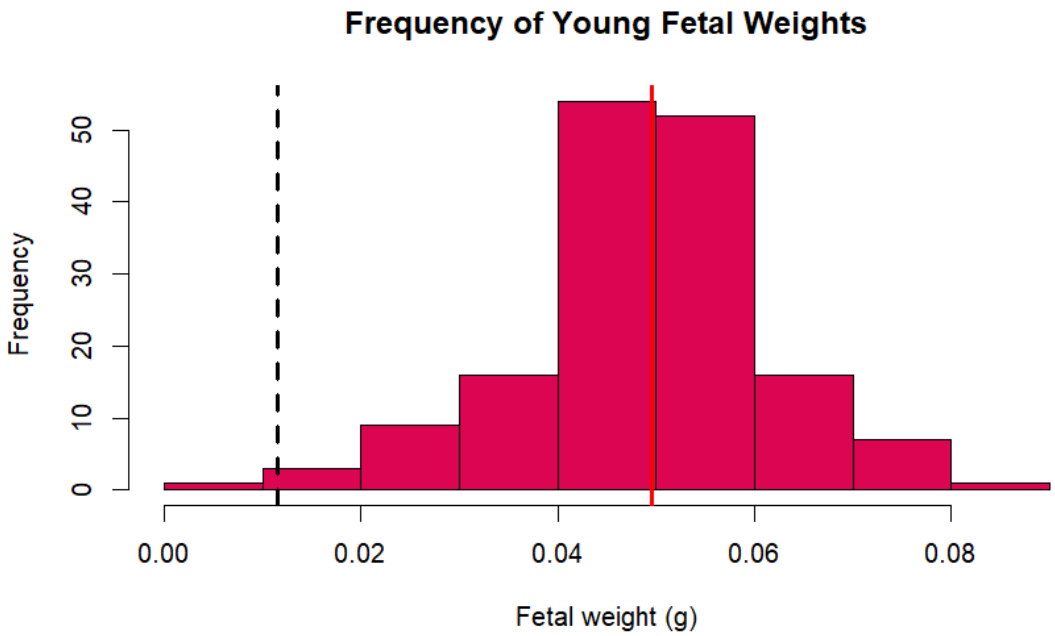


Figure 12. **Frequency of fetal weights in the Young group. The red line represents the mean, and the black dotted line represent the mean minus 3 SD.**

3.5.4. Viable and Unviable Fetuses

The number of viable fetuses was lower, and the number of unviable fetuses was higher in Aged females of both groups using the p-value adjusted for multiple comparisons (unviable fetuses: Aged vs Young: p.adj < 0.0001 & Aged/Exercised vs Young: p.adj < 0.001; Fig. 13; viable fetuses: Aged vs Young: p.adj < 0.001 & Aged/Exercised vs Young: p.adj < 0.0001; Fig. 13), but there was no difference in either trait between Aged and Aged/Exercised females.

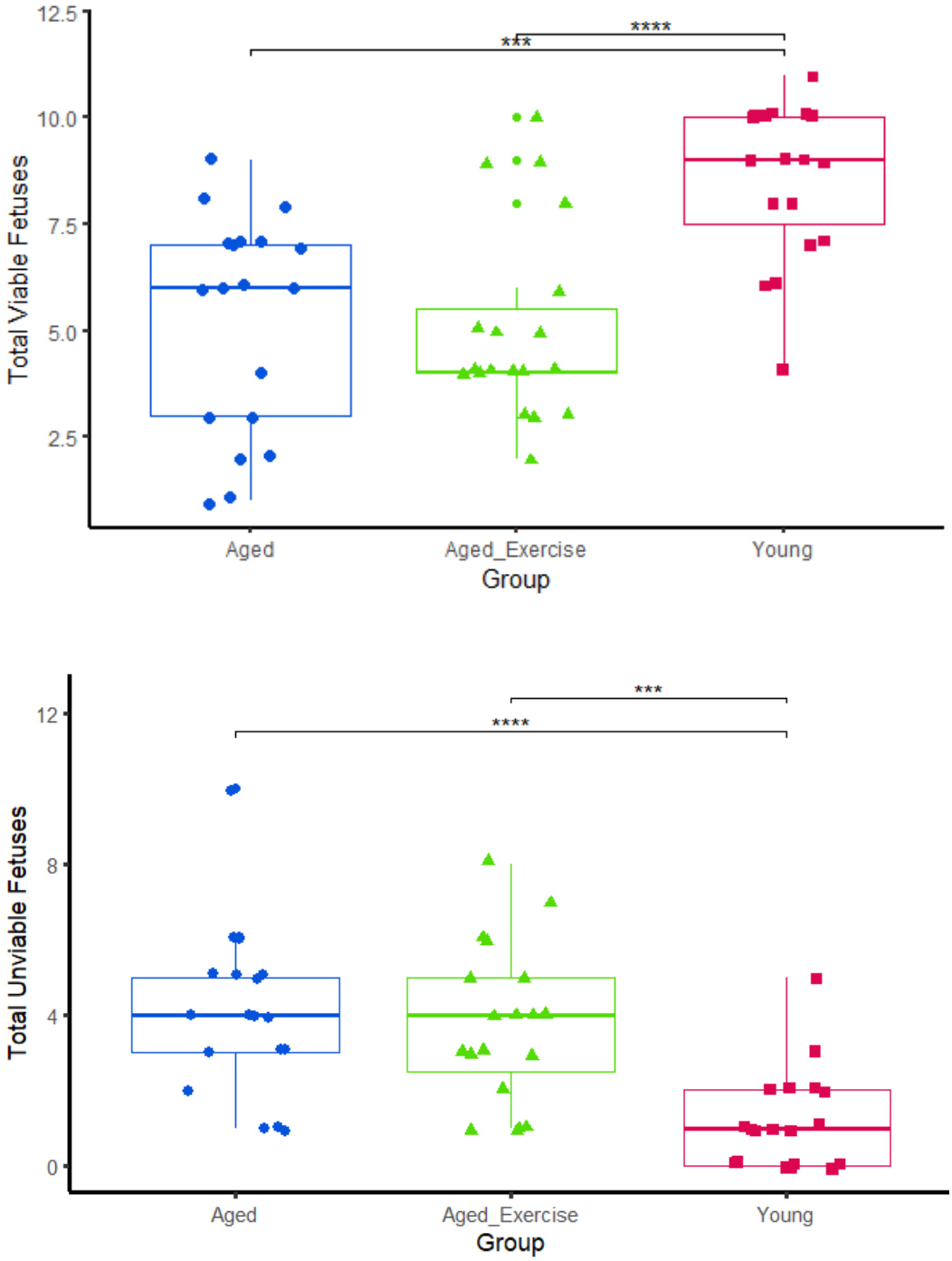


Figure 13. **Viable fetuses and resorptions for the Aged, Aged/Exercised and Young groups. For the viable fetuses (Up) and unviable fetuses (Down), there is a significant overall difference among groups, and pairwise differences identified by the Tukey test show a difference between the Young and Aged/Exercised and Young and Aged groups: p.adj < 0.01: **, p.adj < 0.001: ***, p.adj < 0.0001: ****.**

3.6. Group Differences in Fetal Development Parameters

I assessed whether there were group differences in measures of fetal development: crown–rump length, the width of the head, the cross-sectional area of the body in profile, eyespot pigmentation, presence of digits and fetal weight. I used the average of all the fetuses of each female. The data on crown–rump length, eyespot pigmentation, the body's cross-sectional area of the body and the width of the head violate the assumption of homogeneity of variances, so I performed a Welch's ANOVA test since the data follow a normal distribution.

The Crown–rump length, fetal weight, fetal profile area, width of the head, and eyespot pigmentation were significantly lower in the Aged/Exercised compared to the Young group (p-value ≤ 0.01, Figure 14 A-E). There were no differences between the groups in the development of digits based on the ANOVA (p-value > 0.05, Fig 14).

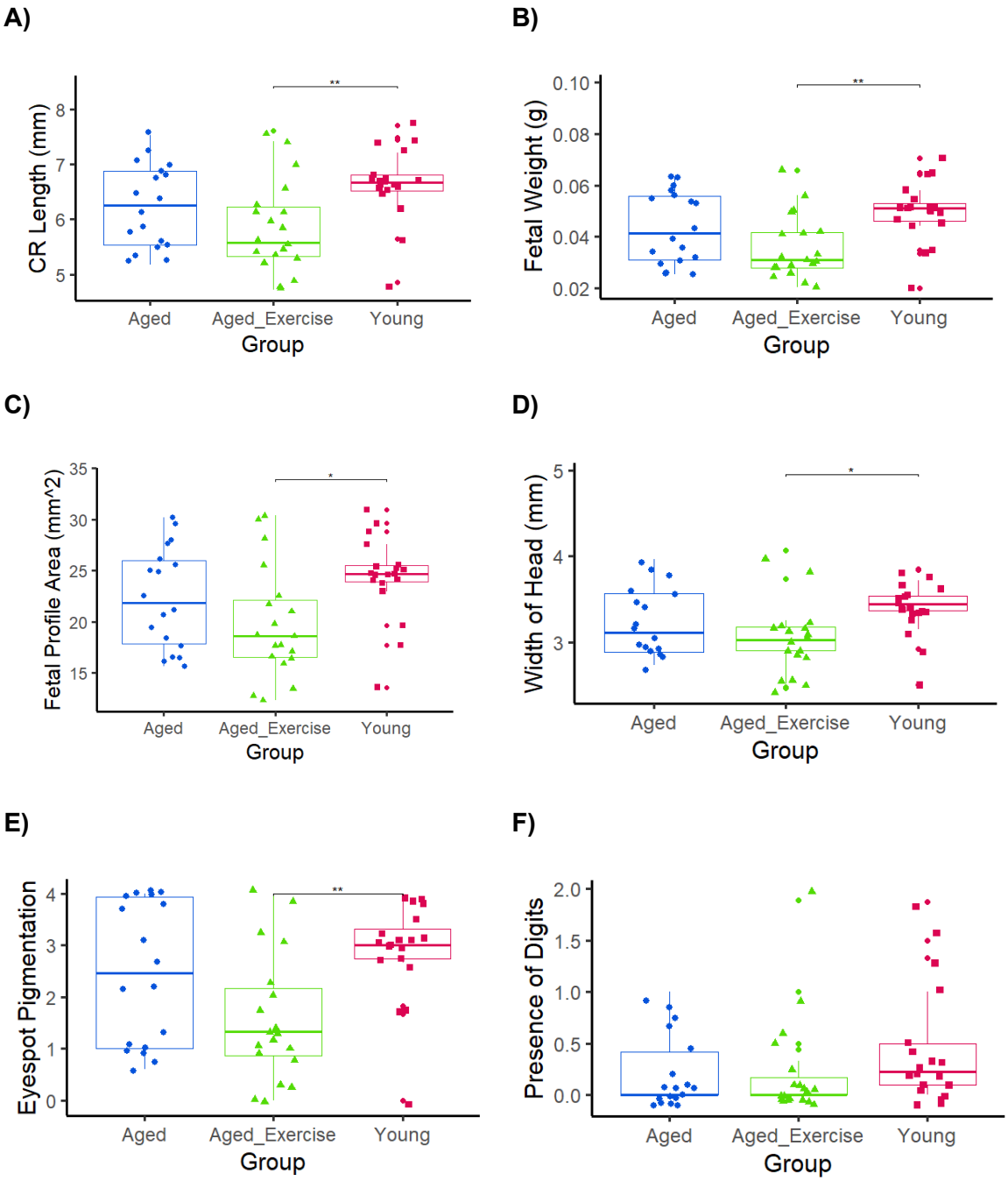


Figure 14. **Fetal development parameters for the Aged, Aged/Exercised and Young groups. A) CR length, B) Fetal weight, C) Cross-sectional area of the body, D) Width of the head, E) Distribution of average eye spot pigmentation and F) Distribution of average presence of digits. Pairwise differences reflect Bonferroni multiple pairwise-comparison tests for Welch ANOVA or Tukey multiple pairwise-comparison tests for ANOVA. $p < 0.05$: *, $p < 0.01$: **.**

3.7. Group Differences in Fetal Development Parameters by Sex

I performed a two-way ANOVA to examine the effects of treatment, fetal sex and the interaction between treatment and fetal sex on fetal development parameters: crown–rump length, the width of the head, fetal weight and the cross-sectional area of the body. Although sex is missing for some fetuses, I used the average of the parameters per female, per sex. CR Length showed significance in the interaction term between the groups and Sex (p-value < 0.05) with the Aged/Exercised female fetuses having lower CR Lengths than male fetuses, and no sex differences in other groups (Figure 15A, p-value < 0.01). There was no significant interaction between Sex and Group for the Presence of digits or Fetal weight, but for the Area, Eyespot pigmentation and Width of the head the interaction term showed a tendency towards significance with the p-value for the Sex:Group interaction = 0.06, again with smaller females only in the Aged/Exercised group (Figure 15 C-E). To analyse the proportion of female and male fetuses I studied the sex ratio between the groups and found no significant difference (Figure 16, p-value > 0.05).

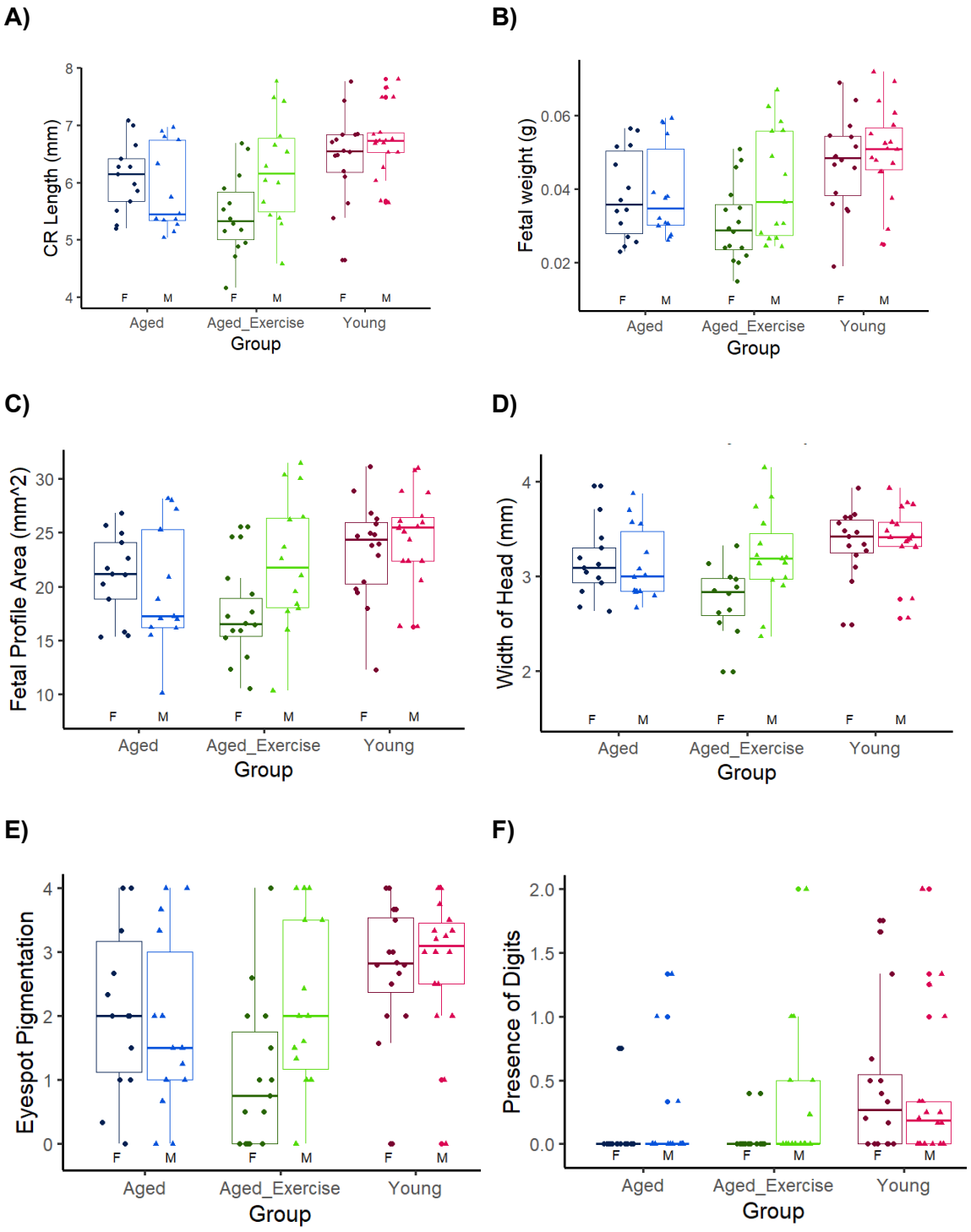


Figure 15. **Fetal development in the Aged, Aged/Exercised and Young groups by sex. A) Crown-rump length, B) Fetal weight, C) Area and D) Width of the head.**

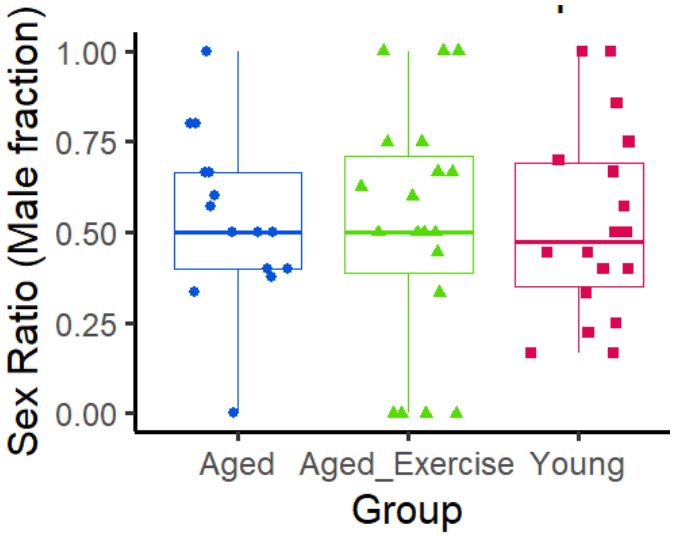


Figure 16. **Sex Ratio shown as the male fraction in each litter (Males / (Males + Females) for the Aged, Aged/Exercised and Young groups.**

3.8. Differential Expression Analysis

I performed global transcriptomic analysis (RNA-sequencing) for the Young, Aged and Aged/Exercised groups. There were no significant interactions between sex and group or differences between the Aged and Aged/Exercised groups (Figure 17A). However, the Aged and Aged/Exercised groups differed from the Young group (p-adj < 0.05). I found 515 deregulated genes between Young and Aged, out of which 181 were more highly expressed, and 334 were downregulated in Aged females. Likewise, I found 786 deregulated genes between the Young and Aged/Exercised groups, of which 306 were more expressed and 480 were down-regulated in Aged females (Figure 18).

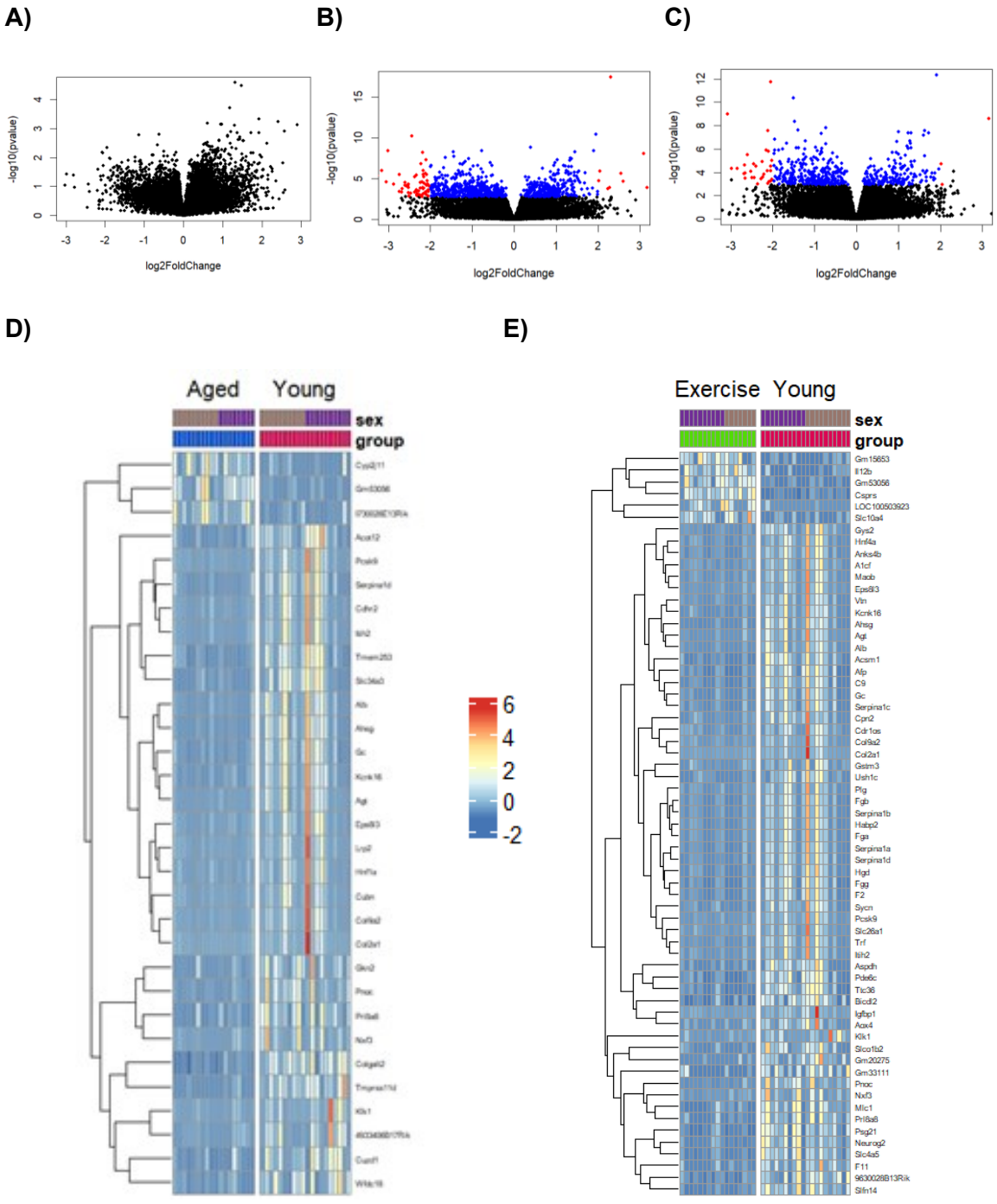


Figure 17. **Differential volcano plots from the GD11.5 placental RNA for the A) Aged vs Aged/Exercise, B) Young vs Aged/Exercised and C) Young vs Aged groups. And heatmaps of the D) Young vs Aged groups and E) Young vs Aged/Exercise. All plots show padj < 0.05, and the heatmaps used log2FoldChange > 2 to show the top genes only. There is no Aged vs Aged/Exercised heatmap since no genes were differentially expressed.**

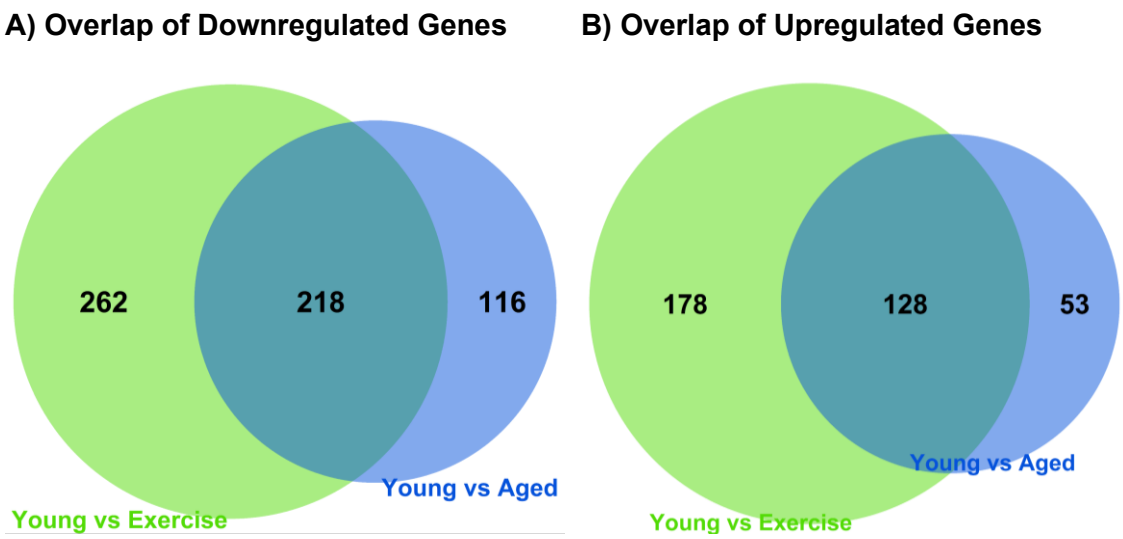


Figure 18. Venn diagram showing the overlap of deregulated genes between Aged - Young and Aged/Exercised - Young. A) Overlap of downregulated genes and B) overlap of upregulated genes. In both cases, the Young group was the control group.

To examine whether the placentas of the Aged groups showed delayed development compared to the Young placentas at GD11, I also examined Young placentas at GD10. The analysis showed that GD10.5 vs Young had 117 upregulated and 5 downregulated genes (Figure 19). In contrast, the Aged/Exercised and Aged groups had 1 upregulated each, and the Aged also showed 3 downregulated genes compared with the GD10.5 Young group. I also analysed the distribution of the top 500 differentially expressed genes between the Aged, Aged/Exercised, Young GD11.5 and Young GD10.5 group across the principal component 1 (PCA1) based on RNA-seq data. The analysis showed a similar distribution between the Aged and Aged/Exercised data points and a closer clustering of both age groups to the Young DG10 than to the Young GD11.5 (Figure 20).

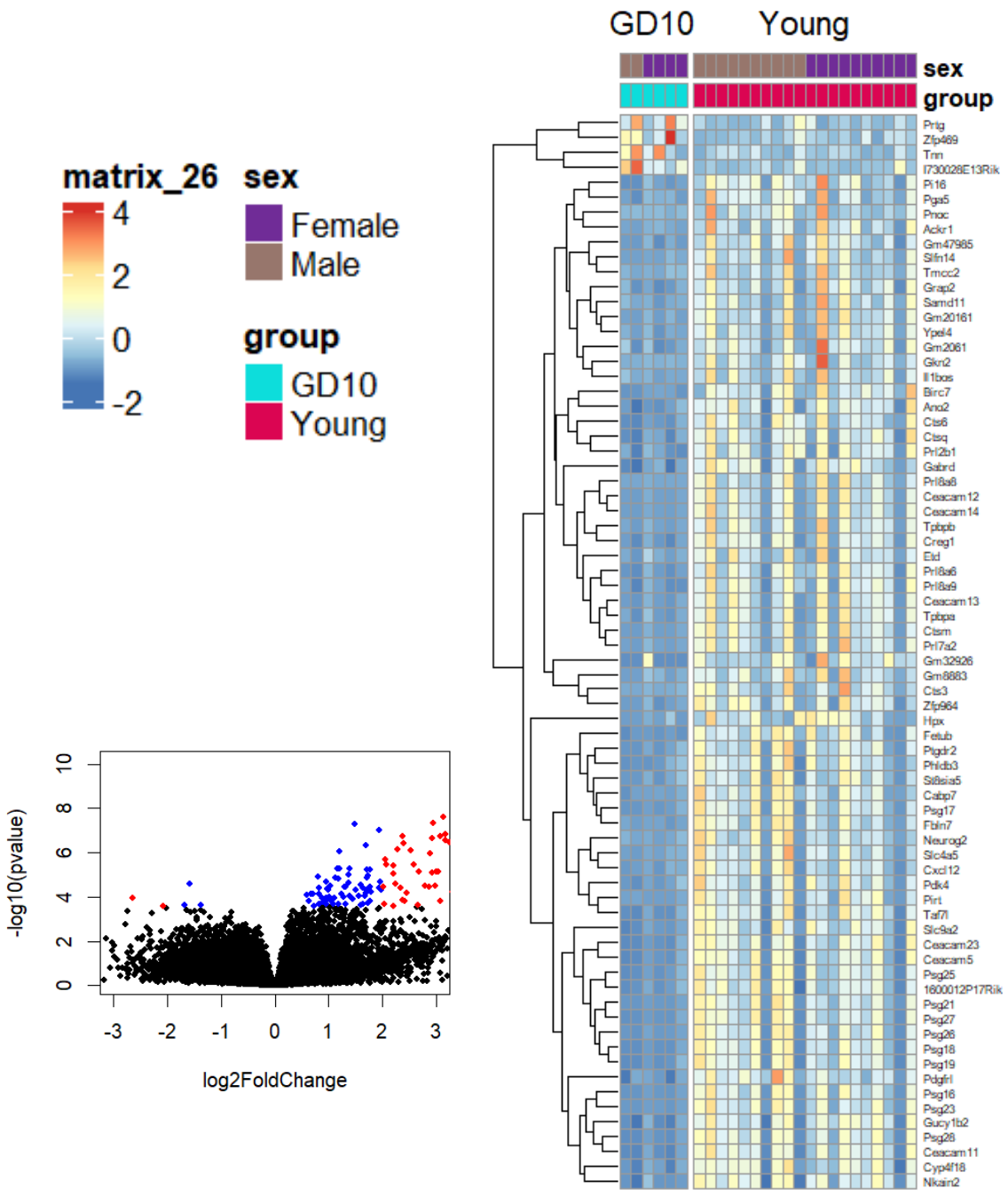


Figure 19. **Differential volcano plots for the Young at GD11.5 vs Young at GD 10. Plots show p-adj < 0.05, and the heatmaps used log2FoldChange > 1.5 to show the top genes only.**

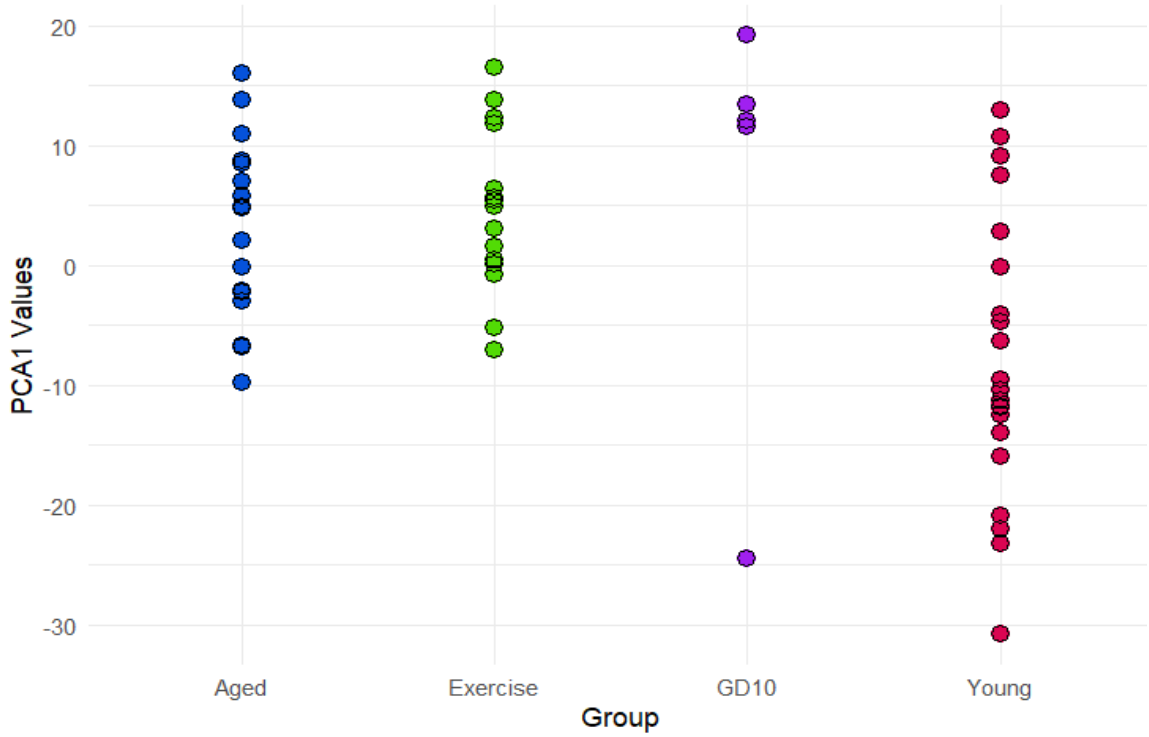


Figure 20. Plot illustrating the distribution of samples across the principal component 1 (PCA1) based on RNA-seq data top 500 differentially expressed genes between the 4 groups: Aged, Aged/Exercised, Young GD11.5 and Young GD10.

To validate the results, I compared the enriched biological pathways obtained from the genes found deregulated in the Age and Aged/Exercised groups compared with a list of deregulated genes previously found by Woods et al. (2017) from GD11.5 decidua deregulated with age, associated with both normal and abnormal fetuses in their study. I found 103 deregulated genes in common between Woods et al and the Aged vs Young analysis in this study, and 125 genes similarly deregulated with the Young vs Aged/Exercised group, including key regulators of decidual differentiation, such as *Bmp2*, Gdf10, Ptger3 and Igf1 for both comparisons. For the enrichment analysis I found 20 overlapping biological processes between the three data sets and 165 enriched biological processes only between the Aged and Aged/Exercised genes but not the Aged Woods data (Figure 21 - 22).

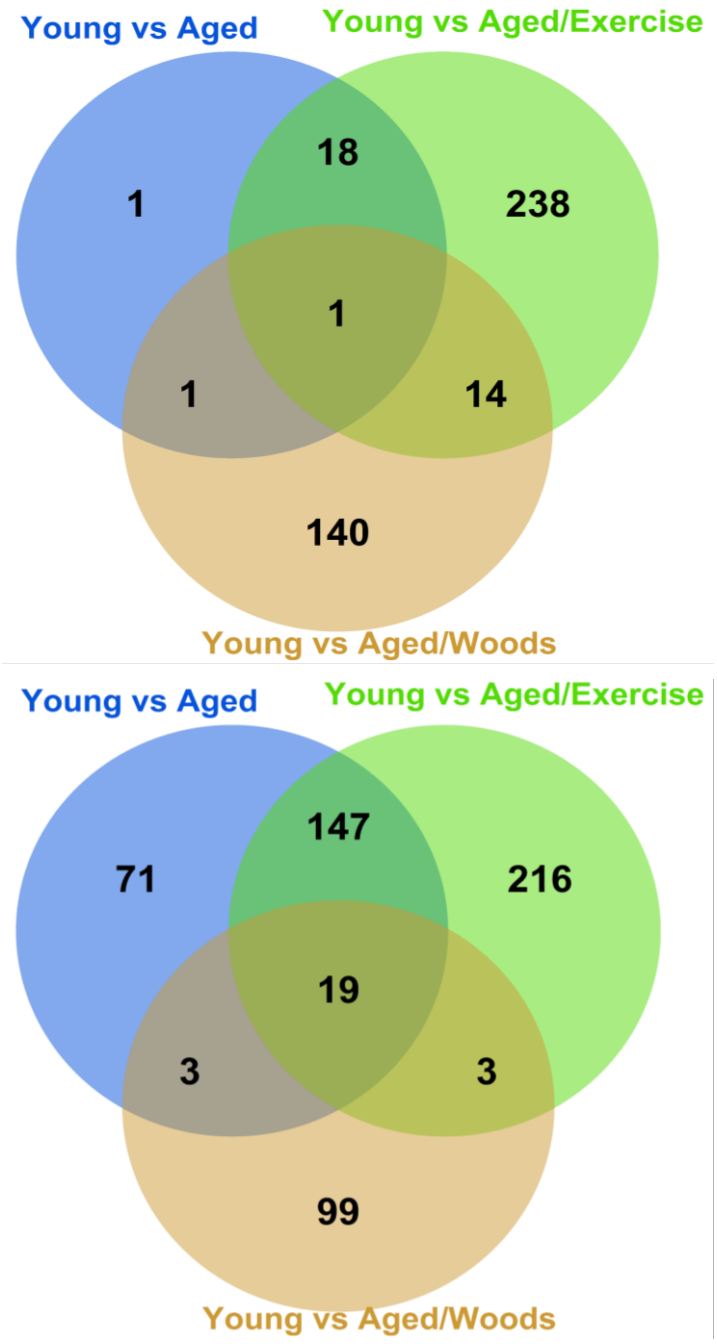
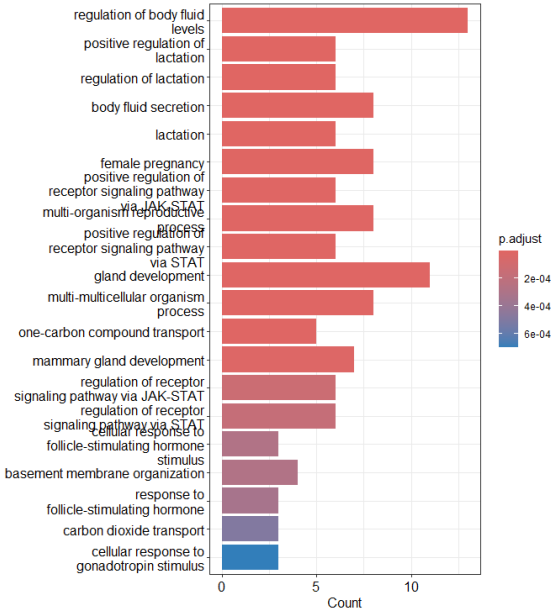


Figure 21. **Venn diagram showing the overlap between Upregulated (Up) and the Downregulated (Down) biological processes enriched in the Aged placenta, the Aged/Exercised placenta, and the Aged decidua from the Woods et al. gene expression data set, all compared to a Young control.**

A)


regulation of body fluid levels
positive regulation of lactation
regulation of lactation
body fluid secretion
lactation
female pregnancy
positive regulation of receptor signaling pathway via JAK-STAT
multi-organism reproductive process
positive regulation of receptor signaling pathway via STAT
gland development
multi-multicellular organism process
one-carbon compound transport
mammary gland development
regulation of receptor signaling pathway via JAK-STAT
regulation of receptor signaling pathway via STAT
cellular response to follicle-stimulating hormone stimulus
basement membrane organization
response to follicle-stimulating hormone
carbon dioxide transport
cellular response to gonadotropin stimulus
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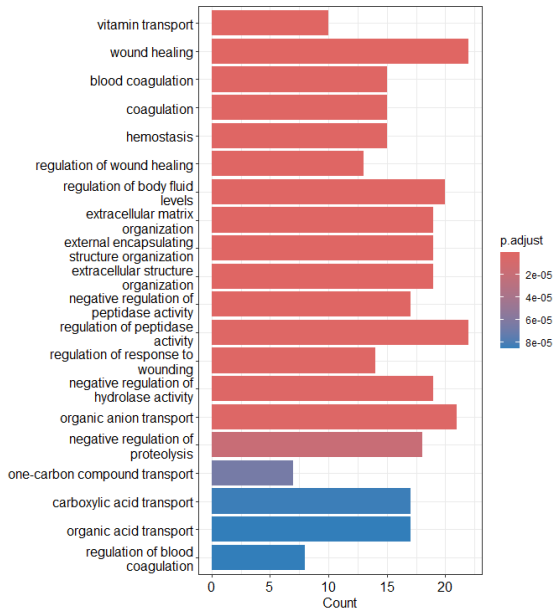
5
10
Count
p.adjust
2e-04
4e-04
6e-04

B)


vitamin transport
wound healing
blood coagulation
coagulation
hemostasis
regulation of wound healing
regulation of body fluid levels
extracellular matrix organization
external encapsulating structure organization
extracellular structure organization
negative regulation of peptidase activity
regulation of peptidase activity
regulation of response to wounding
negative regulation of hydrolase activity
organic anion transport
negative regulation of proteolysis
one-carbon compound transport
carboxylic acid transport
organic acid transport
regulation of blood coagulation
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5
10
15
20
Count
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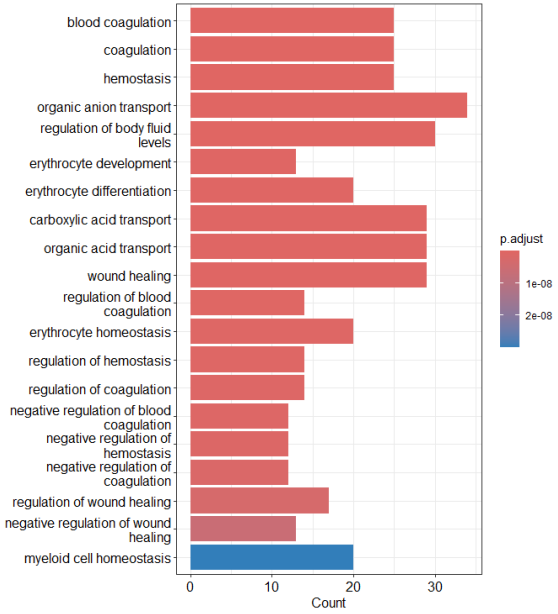
2e-05
4e-05
6e-05
8e-05

C)


blood coagulation
coagulation
hemostasis
organic anion transport
regulation of body fluid levels
erythrocyte development
erythrocyte differentiation
carboxylic acid transport
organic acid transport
wound healing
regulation of blood coagulation
erythrocyte homeostasis
regulation of hemostasis
regulation of coagulation
negative regulation of blood coagulation
negative regulation of hemostasis
negative regulation of coagulation
regulation of wound healing
negative regulation of wound healing
myeloid cell homeostasis
0
10
20
30
Count
p.adjust
1e-08
2e-08

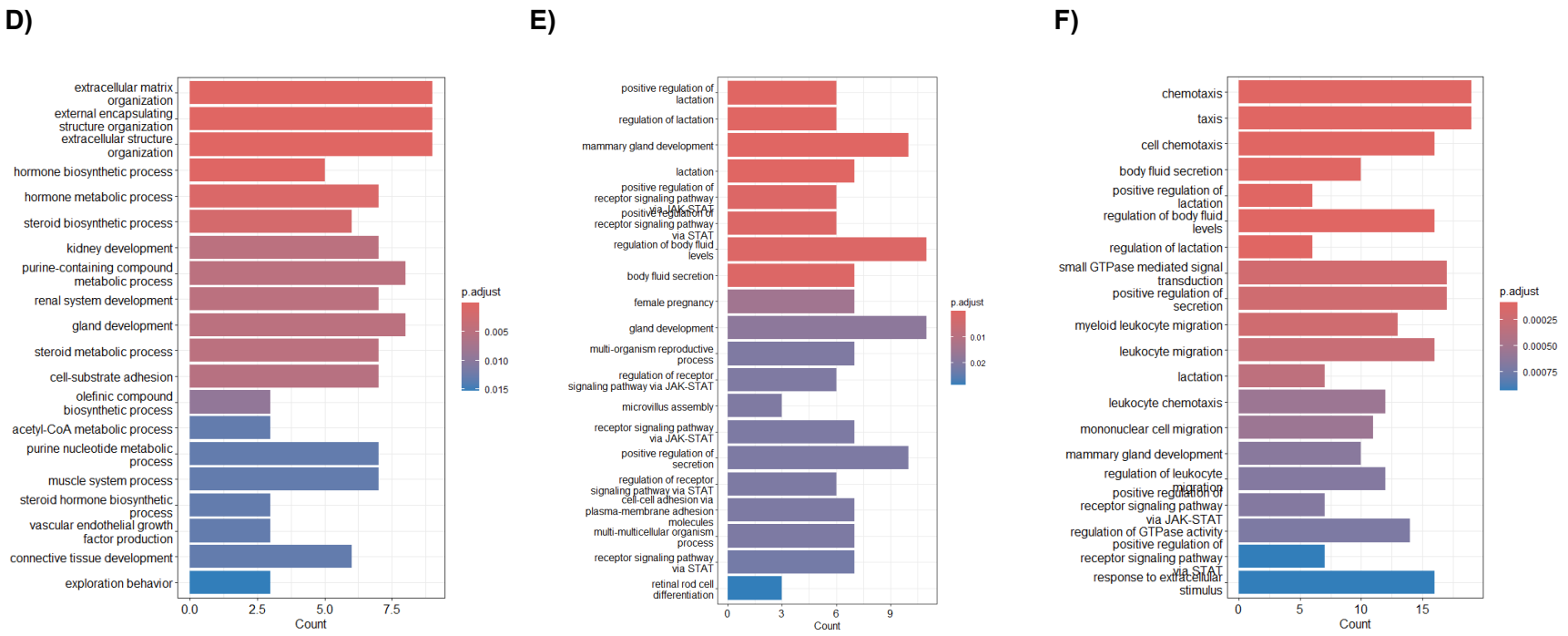


Figure 22. **Gene enrichment analysis showing biological processes for the downregulated: A) Young vs Aged from the Woods et al. data set, B) Young vs Aged, C) Young vs Aged/Exercised, and upregulated: D) Young vs Aged from the Woods et al. data set, E) Young vs Aged and F) Young vs Aged/Exercised.**

3.9. Differences in fetal parameters from the samples used for RNA-seq

Because the RNA was extracted only from normal-looking placentas from each group, I analyzed the developmental parameters for those corresponding fetuses with the CR Length, Fetal Weight, Width of the head and Fetal Profile area, and only the Fetal Weight remained significantly different between the Aged/Exercised group and the Young group, i.e., our sample of placentas for RNA-seq was somewhat biased towards larger fetuses.

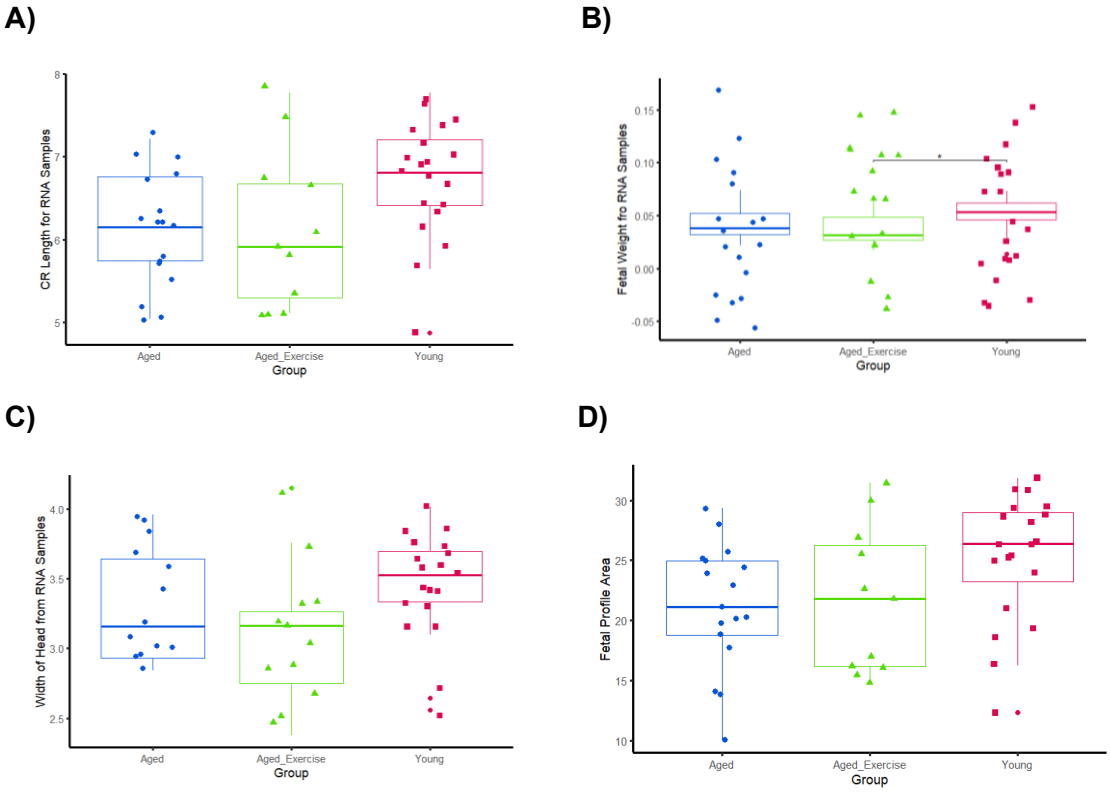


Figure 23. Fetal development parameters in the Aged, Aged/Exercised and Young groups for the sample used for RNAseq processing. A) Crown-rump length, B) Fetal weight, C) Width of the head and D) Area.

3.10. Deconvolution

I used the BisqueRNA package to estimate cell type proportions in the placental RNA-seq data for the four groups: Aged, Aged/Exercised, Young, and Young GD10.5. I used two single-cell data sets as a reference: the “AllNuclei” data, including all placental cell types, and “TrophoblastNuclei” data, including only different trophoblast types, published by Marsh and Blelloch. I performed deconvolution using each data set separately. I analyzed the difference between groups for each cell type by ANOVA, plotting the significant results in Figure 26. The trophoblast cell analysis revealed significantly fewer Junctional zone precursor 2 cells in the Aged/Exercised group than in the Aged group, significantly higher Sinusoidal trophoblast giant cells precursor cells in the Young GD10.5 than in the GD11.5, and a higher proportion of Spongiotrophoblast cells in the Young GD11.5 than in the three other groups (Fig. 24 and Fig. 26 A-C). The “AllNuclei” analysis revealed significantly fewer endothelial cells in the Young GD11.5 than in the Young GD10.5 (Fig. 25 and Fig. 26 D). No significant difference in the proportion of blood cells, decidual stroma, or fetal mesenchyme was found between the groups (Supplementary Figure A1).

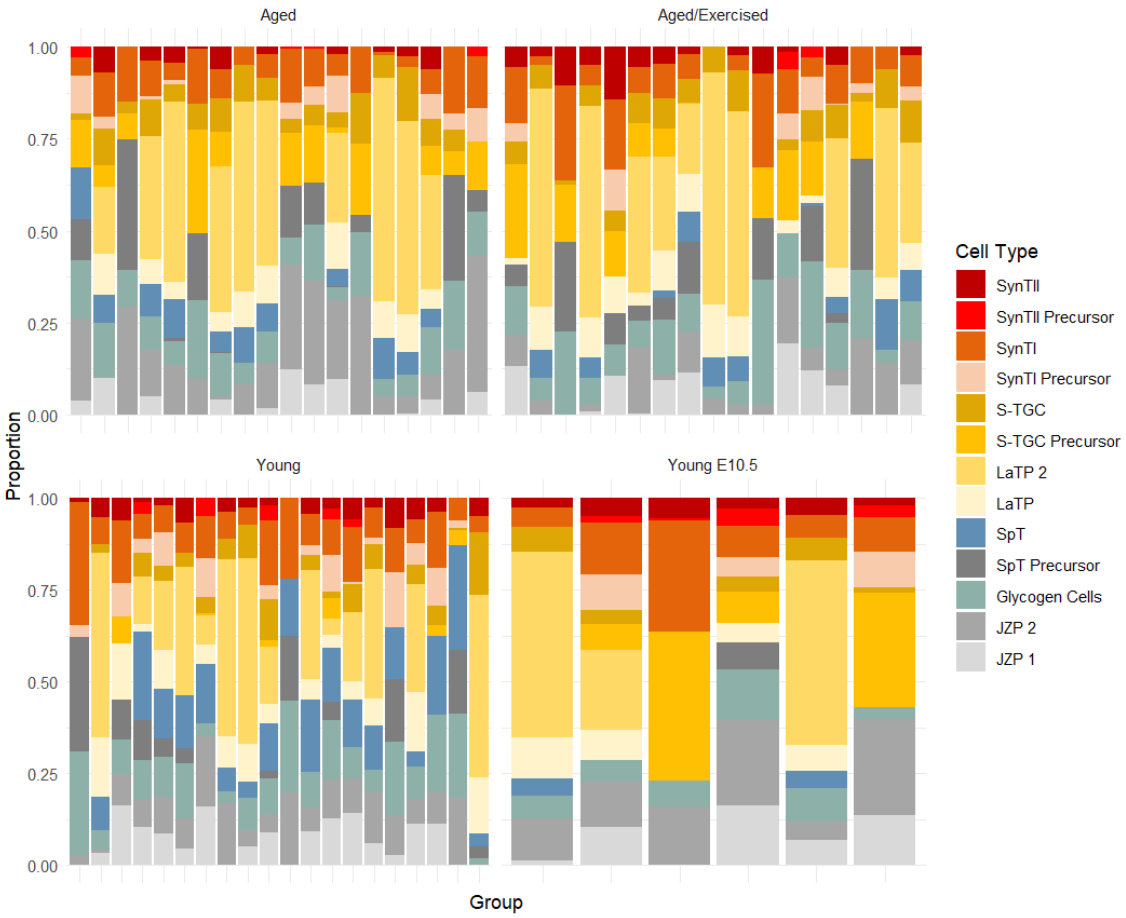


Figure 24. **Virtual deconvolution of placental RNA-seq data for cell type-specific composition using snRNA-seq reference data of 13 trophoblast cell type clusters at GD10.5 and E12.5 obtained from Marsh and Blelloch 2020. Each vertical bar represents an individual placenta, with proportional cell type composition depicted. LaTP = Labyrinth precursor, JZP = Junctional zone, SynT = Syncytiotrophoblast, S-TGC = Sinusoidal trophoblast giant cells, SpT = Spongiotrophoblast.**

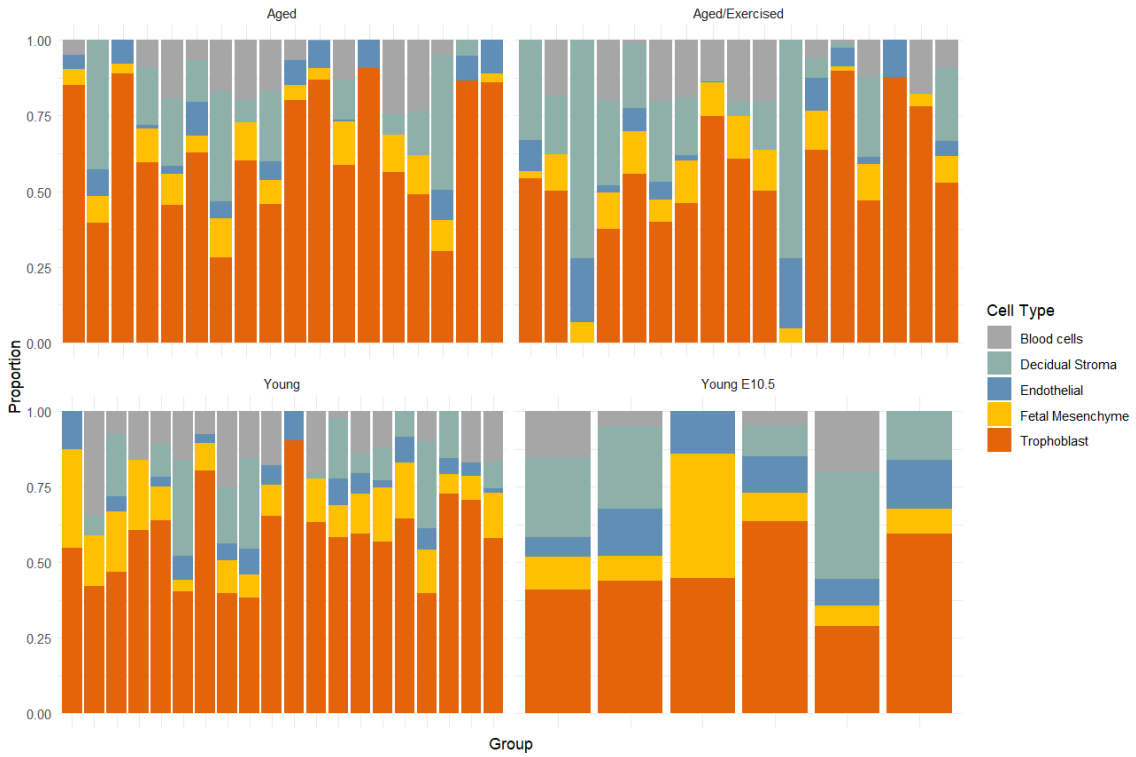


Figure 25. **Virtual deconvolution of placental RNA-seq data for cell type-specific composition using snRNA-seq reference data of 5 cell types clusters at GD10.5 and E12.5 obtained from Marsh and Blelloch 2020. Each vertical bar represents an individual placenta, with proportional cell type composition depicted.**

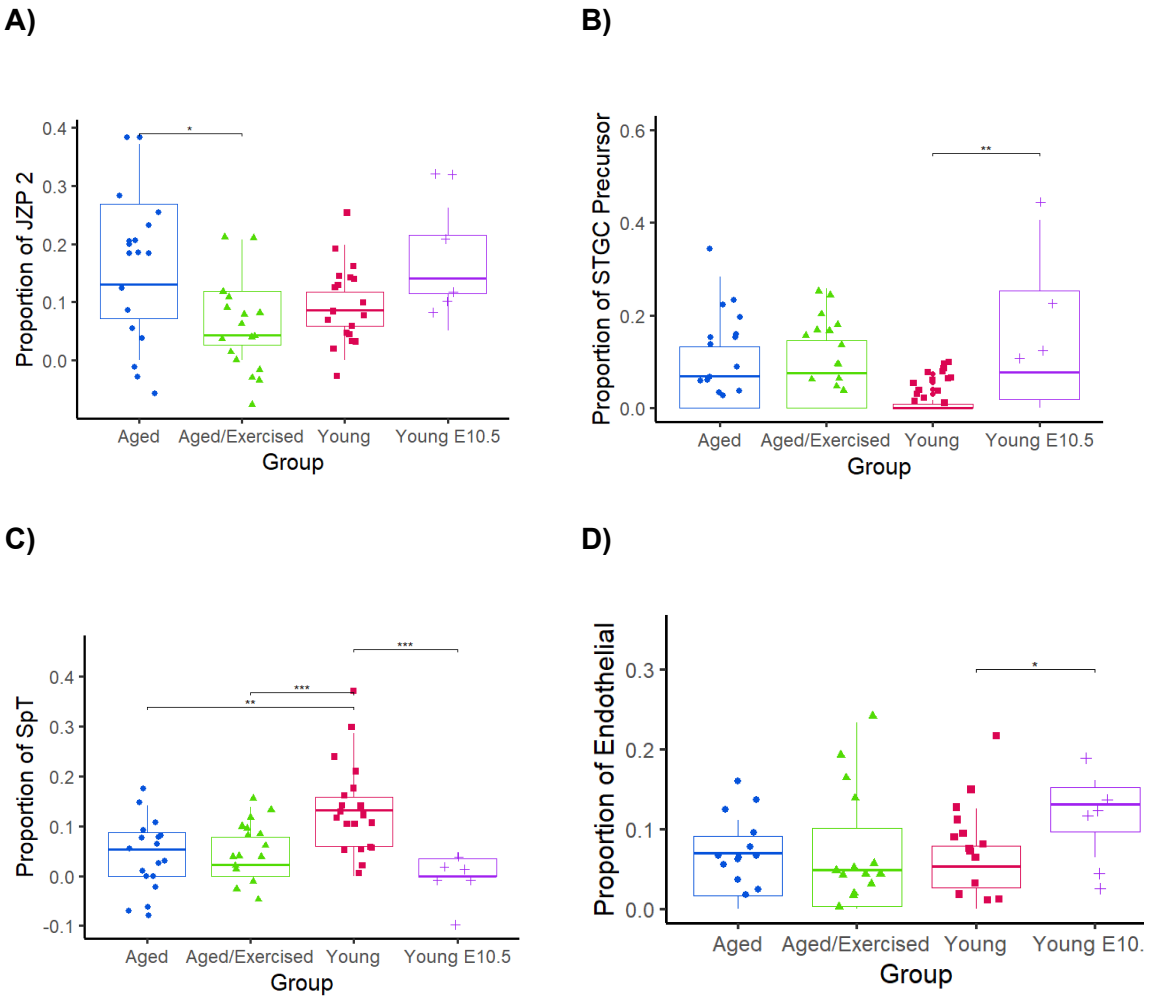


Figure 26. **Statistically significant cell type differences from the virtual deconvolution of placental RNA-seq. Statistically significant differences based on ANOVA from TrophoblastNuclei deconvolution: A) Junctional zone precursor 2, B) Sinusoidal trophoblast giant cells precursor and C) Spongiotrophoblast. Statistically significant difference based on ANOVA from AllNuclei deconvolution: D) Endothelial cells. p.adj < 0.05: *, p.adj < 0.01: **, p.adj < 0.001: ***.**

Chapter 4. Discussion

4.1. Does exercise mitigate advanced maternal age's effects on pregnancy outcomes in a mouse model?

Advanced maternal age is associated with an increased risk of pregnancy complications such as pre-eclampsia, stillbirth and fetal growth restriction. In rodent models, advanced maternal age has been associated with an increased resorption rate, embryonic malformation and delay and placental abnormalities such as increased thickness of the trophoblast giant cell layer (Lopes et al. 2009; Woods et al. 2017).

A minimum of 150 min each week of moderate-intensity aerobic physical activity is recommended for pregnant women, according to the World Health Organization ("WHO Guidelines on Physical Activity and Sedentary Behaviour," n.d.). Exercise during pregnancy is associated with lower risks of pre-eclampsia, gestational hypertension, gestational diabetes, excessive gestational weight gain and delivery complications (Paulsen et al. 2023; Gascoigne et al. 2023). I therefore hypothesized that maternal exercise would improve fetal growth and alter placental gene expression in a mouse model of advanced maternal age. The reproductive performance of the Aged/Exercised females was similar to that of the Aged females, with no significant differences in the number of viable or unviable fetuses in their litters. Similarly, there was no difference in fetal parameters or placental gene expression between the Aged/Exercised and Aged females, regardless of fetal sex. However, some of the parameters show that fetal growth and development were significantly reduced in the Aged/Exercised compared to the Young group. Overall, the data show that an exercise treatment does not help mitigate the effects of advanced maternal age in a mouse model.

4.2. Age and Exercise in Pregnancy Outcomes and Fetal Development

The Aged/Exercised females had 1.4 fewer conceptuses than the Young mice, but no significant difference was found in the number of conceptuses between the Young and Aged females. When studying murine models of pregnancy, the litter size (or total number of conceptuses) is considered a good indicator of fecundity (Dalton-O'Reilly et al. 2023). In this study, only the Aged/Exercised females had a significantly

lower number of conceptuses than the control group and the number of viable fetuses was significantly negatively correlated with the amount of exercise in the Aged/Exercised group, implying that exercise in advanced maternal age mice leads to a decline in reproductive capacity.

Both Aged groups showed fewer viable fetuses (~3 viable fetuses, p-value < 0.001) and a higher number of unviable fetuses (~2 unviable fetuses, p-value < 0.01), including resorptions and small-weight fetuses. This is consistent with previous studies showing that aged pregnancies show a significant increase in the number of resorption sites (Lopes et al. 2009). Resorptions indicate fetal loss post-implantation, either in the early or late stages (Gendron and Baines 1989). In this study, we included the unviable fetuses based on their weight and the conceptuses classified as resorptions from the dissections, so the total unviable fetuses reflect both early and late fetal losses. Even so, the increase of ~2 unviable fetuses is less than the decrease of ~3 in viable fetuses, suggesting that there was also loss during or before implantation and/ or that the number of ovulations was reduced.

Crown–rump length and fetal weight were similar in the Aged and the Aged/Exercised females. For the fetal area, the width of the head and eyespot pigmentation, there was a significant difference only between the Aged/Exercised females and the Young females, with the Aged females intermediate between the Young and Aged/Exercised groups. Advanced maternal age has been consistently associated with embryo abnormalities and our results confirm this while also indicating that the Aged/Exercised fetuses show more severe effects than the Aged fetuses with higher developmental delay and/or malformations (Lopes et al. 2009). McPherson et al. showed that exercise treatment to improve the adiposity in males fed a high-fat diet improved embryo development and implantation capabilities (McPherson et al. 2013). These improvements were not observed with exercise treatment for advanced maternal-age mothers in our study.

4.3. Effect of maternal weight in pregnancy complications

Most of the adverse outcomes associated with advanced maternal age, such as preterm birth, fetal growth restriction and preeclampsia, have also been associated with overweight or obesity (Waldenström et al. 2014; Lewandowska et al. 2020). Obesity is

characterized as excess adipose tissue and is clinically characterized by body mass index (BMI) > 30 kg/m^2. A high body mass index (BMI) and excessive gestational weight gain (GWG) are associated with adverse pregnancy outcomes such as increasing risks of macrosomia, postpartum hemorrhage, cesarean section, gestational diabetes mellitus and preeclampsia or arterial hypertension (J. Zhang et al. 2023; Krsman et al. 2023; Ke et al. 2023). Obesity can alter placental function and its ability to transport nutrients due to inflammation, oxidative stress, insulin resistance and dysregulation of cytokines (Waker et al. 2023; Lewandowska 2021). We observed a significant reduction in both the pre-pregnancy weight and GWG in the Aged/Exercised females compared to the Aged females which allowed us to examine the effects of obesity in the pregnancy outcomes.

The weight at the time of mating (pre-pregnancy) was significantly higher in the Aged/Exercised females than in the Young females, however, there was no significant difference between the Aged/Exercised females and the Young females in the weight gain at time of collection without the uteri, indicating that the exercise treatment regulated weight gain even though the weight at the time of mating was significantly higher. Although the weight gain in the Aged females was significantly higher comparing to the Aged/Exercised females, it did not reach significance compared to the Young females with a p-value = 0.08. The consequences of excessive GWG and overweight and obesity in adverse pregnancy outcomes have been inconsistent. While some studies show that excessive GWG is associated with adverse outcomes such as gestational diabetes, preeclampsia, spontaneous preterm birth, and fetal macrosomia regardless of the pre-pregnancy BMI, others have found that pre-pregnancy obesity has a greater impact on these adverse outcomes compared to excessive GWG (Lewandowska 2021; Gascoigne et al. 2023). Ke et al., found that maternal pre-pregnancy overweight and obesity are more strongly associated with maternal complications while excess GWG is more associated to fetal complications in GDM women in China (Ke et al. 2023). Although we expected that reducing the weight in the Aged/Exercised group would improve the pregnancy outcomes related to overweight and excess GWG, there was still an adverse effect on the pregnancy outcomes compared to the control, which could suggest the effect is related specifically to maternal age and not to adiposity.

4.4. Sex differences in fetal parameters and placental transcriptome

While most fetal developmental parameters showed a significant difference between Aged/Exercised and Young fetuses, only CR Length showed a significant interaction between groups and sex. Area, Eyespot pigmentation and Width of the head showed a marginally non-significant interaction as well. There is a trend where the female fetuses are smaller than male fetuses, but only in the Aged/Exercise group.

Several studies show sexual dimorphisms that impact both fetal and maternal health outcomes in pregnancy. Fetal growth is affected by sex; males are generally heavier at birth, with macrosomia more common in males, while females are more likely to be growth reduced (Flowers et al. 2024; Clifton 2010). These differences in fetal growth are also observed in adverse environments during pregnancy, such as pre-eclampsia and preterm delivery, suggesting male and female fetuses have different mechanisms to cope with adverse environments or stress. It has been suggested that males adopt a strategy that allows them to continue to grow normally in an adverse environment, such as asthma or preeclampsia, while females adapt by reducing their growth; this results in placing males at risk in the presence of a second stressful event and making them more susceptible to stillbirth and preterm birth, while making the female fetuses able to adapt and reduce the effects that stress or different environmental conditions may provoke in the pregnancy (Clifton 2010). This proposed strategy is not always observed and might be not consistent throughout gestation (Hercus, Metcalfe, and Christians 2024).

Even though advanced maternal age is associated with many adverse pregnancy outcomes and, therefore, could be considered an adverse intrauterine environment, no significant difference in fetal growth between the sexes was found, although this lack of sex difference might be due to the gestational stage, while I studied GD11.5, most studies considering sexual dimorphisms are at term. No significant difference between the sexes can be observed for the other fetal parameters of development, except for CR Length. Aged/Exercised females appear to have a tendency towards lower CR-length, fetal weight, profile area, width of the head and eyespot pigmentation than the Aged/Exercised males and the other females or males in the other groups. This suggests that the females might have been more affected than

the males by the exercise treatment. However, only the CR length was significant. No differences in sex ratio were observed either, indicating no sex differences in survival.

The placenta is the organ responsible for moderating fetal growth by mediating nutrient and oxygen exchange. It is, therefore, possible that dimorphic changes in the placenta may contribute to the difference in fetal growth and pregnancy outcomes observed between males and females (Kalisch-Smith et al. 2017). The size of the labyrinth and junctional zone of aged female rat placenta increases compared to the young placenta. There is also upregulation of the genes Igf2 and Prl3b1 in female placentas and a decrease of IFG2 protein in male placentas (Napso et al. 2019). However, the data presented by Napso et al. were analyzed separately for each sex, rather than testing for the interaction between sex and maternal age, which might be the reason for the difference in results. I found no differentially expressed genes significant between the sexes for any of the groups when testing sex by group interactions. Altogether the results show that sexual dimorphism was not consistently observed.

4.5. Genes differentially expressed in placentas of mouse models of advanced maternal age

The placenta is a crucial organ during pregnancy and regulates communication between the fetus and the mother. In mice, the placenta has three main regions, the decidua, the maternal uterine tissue that protects and supports the nutrition of the embryo; the junctional zone that secretes hormones; and the labyrinth, where nutrient exchange occurs between fetal and maternal blood. My study examined the whole placenta at GD11.5, but Woods et al. did a transcriptomic analysis of advanced maternal age in mouse decidua only at GD11.5. Comparing our results with those of Woods, there were 103 similarly deregulated genes in common with the Aged placentas and 125 deregulated genes in common with the Aged/Exercised placentas, including key regulators of decidual differentiation, such as Bmp2, Gdf10, Ptger3 and Igf1.

Bone morphogenetic protein family (Bmp2) is a growth factor belonging to the transforming growth factor (TGF) superfamily. Bmp2 is initially detected in the decidual stroma surrounding the site of blastocyst attachment and is known for regulating the proliferation and differentiation of the uterine lining during implantation. *Bmp2* suppression compromises endometrial stromal cell differentiation, which is required for

decidualization (Lee et al. 2007). *Bmp2* was upregulated in the Aged/Exercised group. Deng et al demonstrated that the upregulation of Bmp2 could be a compensation for a poor trophoblast invasion in humans (Deng et al. 2023). Despite *Bmp2* regulating the trophoblast invasion and function, Aged/Exercised pregnancy outcomes were similar to the Aged fetuses suggesting that even though there was an increase in genes related to decidualization there was no improvement in fetal development at GD11.5.

Igf1 was upregulated in both aged groups in the present study and in the aged group from the Woods et al. study. IGF-I is estrogen-induced and produced in endometrial cells (Inoue, Takeuchi, and Takahashi 2005). IGF-I stimulates fetal growth when nutrients are available and has been shown to modify placental nutrient utilization and transfer but not placental size (Fowden 2003). However, the fetal size was lower in both of the Aged groups and in the advanced maternal age model from Woods et al. study, suggesting that the upregulation of *Igf1* may have been a compensatory response. It is possible that fetal growth would have been even more impaired had *Igf1* not been upregulated.

4.6. Enriched Biological pathways

Since the RNA seq for the Aged and Aged/Exercised groups was from the whole placenta, I analyzed the biological pathways enriched in the overlapping differentially expressed genes between both data sets and the differentially expressed genes in the Aged deciduas collected by Woods et al. I also examined the differentially expressed genes overlapping between the Aged and Aged/Exercised groups but not with the genes from the Aged deciduas from Woods data.

The most significant downregulated pathways shared between the three groups include coagulation, haemostasis, and positive regulation of receptor signalling pathway via JAK-STAT, suggesting that these biological pathways are consistently downregulated due to the advanced maternal age of the mothers. The signal transducer and activator of transcription 3 is associated with activating Pgr target genes, which initiates decidualization. This suggests that the downregulation of the signalling pathway JAK-STAT might reduce the availability of Stat3, leading to increased resorption rates and impairment of proper decidualization. The downregulation of the clotting processes might be due to the downregulation of clotting factors such as F7 and F10. One of the

significantly downregulated genes in the Aged/Exercised group is PROZ, Protein Z, Vitamin K Dependent Plasma Glycoprotein, an anticoagulant protein which at low levels in plasma is associated with thrombotic disorders and other adverse pregnancy outcomes such as preeclampsia (Rivera et al. 2021) .

On the other hand, biological processes overlapping between the Aged and Aged/Exercised groups but not with the Aged group from the Woods et al. data set might help us differentiate between the deregulation of processes in the decidua and the rest of the placenta. Processes deregulated in this study but not that of Woods et al. include upregulation of lactation, mammary gland development, lactation, and female pregnancy due to several genes in the prolactin family, as well as downregulation of vitamin transport, vitamin transmembrane transport, and vitamin D metabolic process. Suggesting that these processes are not deregulated in the aged decidua and might be more present in other parts of the placenta, such as the labyrinth, where maternal and fetal blood exchange nutrients, gases, and waste products. Since vitamin D could be a regulator of the expression of placental amino acid transporters, vitamin D insufficiency might influence fetal growth (Cleal et al. 2015).

4.7. Delayed Development in Aged Placentas

Decidualization from advanced maternal age mouse mothers at ~43 weeks of age resembles an earlier developmental stage by 1 day, meaning that GD11.5 aged decidua resembles GD10.5 deciduas of the young controls (Woods et al. 2017). Although I did not separate the decidual portion of the placenta, I obtained similar results comparing the gene expression from the GD11.5 Aged and Aged/Exercised placentas with the GD10.5 placentas, with the Aged groups resembling the earlier stage in gene expression by showing only 4 differentially expressed genes compared to 123 differentially expressed genes between the Young control at GD11.5 and the young GD10.5 placentas. Other studies have shown delayed development due to factors such as female diabetes, high-fat diet in males and maternal stress (Zhao et al. 2017; McPherson et al. 2013; Choe et al. 2011). Fetuses from stressed mothers not only show reduced weight but also show signs of delayed limb development, including a delay of half a day in the expression of the fibroblast growth factor *Fgf8* by immunoreactivity at GD10.5 in ICR strain mice, a key signalling molecule in limb development (Moon and Capecchi 2000; Choe et al. 2011).

A potential mechanism that could explain the delayed development is the nuclear availability of activated Stat3, which is reduced in stromal cells of aged females, leading to inadequate levels of target gene activation and, therefore, an inappropriate molecular response to the decidualization stimulus since Stat3 interacts with the progesterone receptor A (Pgr-A) (Woods et al. 2017). Stat3 phosphorylation is induced via the Jak/Stat pathway, and the gene enrichment analysis showed downregulation of the receptor signalling pathway via JAK-STAT in both the Aged and Aged/Exercised groups together with the Aged group in the Woods data set, suggesting that the exercise treatment did not ameliorate the decidualization process in advanced maternal age.

Furthermore, the virtual deconvolution analysis revealed a higher estimated proportion of spongiotrophoblast cells in the Young GD11.5 than in the three other groups, again indicating that the Aged and Aged/Exercised groups were more similar to the Young GD10.5 group. Spongiotrophoblast cells comprise most of the cells in the junctional zone, beginning around GD9.5 and increasing by four-fold at the end of gestation (Elmore et al. 2022). Although no significant difference in the proportion of decidual cells was observed (Figure 25, Supplementary Figure 1A), the lower proportion of spongiotrophoblasts in the aged groups, and similar to the Young placentas at GD10.5, might further show delayed development of the Aged and Aged/Exercised placentas. The Aged/Exercised group showed a significantly lower proportion of junctional zone precursor 2 cells than the Aged group, making it more similar to the GD 11.5 group than the GD 10.5, the only instance where treatment with exercise appeared to correct age-related changes.

4.8. Limitations

Physical activity is critical to an animal's survival in its natural environment, where rodents are good climbers and jumpers and require brief and rapid runs to escape predators (Richter, Gass, and Fuss 2014). This behaviour can be adaptive in captivity because it helps them maintain physical condition despite their restricted environment, which is crucial for their health and well-being (Harri et al. 1999). One limitation of the present study was the variation in the amount of exercise due to the selection of voluntary wheel running as the form of exercise. Variation between the mice used for the experiment ranges from 0.35 km to 8.56 km per night between mice and

0.35 km to 5.03 km between days from the same mouse before pregnancy. Also, most of the mice showed a reduction in the amount of exercise during the pregnancy compared to the pre-pregnancy measurements. The level of exercise varies depending on the species and the mouse strain, ranging from 2 to 12 km/day and even between individuals of the same strain or species. For example, variation from 1000 to 12000 wheel revolutions per day was seen in Sprague-Dawley rats (Richter, Gass, and Fuss 2014).

The mouse strain C57BL/6J has been reported to run voluntarily from 4 to 20 km/day during a total activity time of 3 to 7 h per day, covering most of their active phase (12 dark hours), which exceeds common human exercise amounts, limiting its comparability to aerobic exercise in humans (Manzanares, Brito-da-Silva, and Gandra 2018; Biedermann et al. 2016). A method to overcome this obstacle is to do forced exercise for a limited number of hours a day. Biedermann et al., tested this by comparing restricted (voluntary for 6 hours) to unrestricted (24-hour access) and found that restricting the amount of running was associated with more stereotypic behavior. Stereotypic behavior such as twirling and bar-mouthing that may be associated with poor animal welfare suggesting that restricting the amount of running may rather be stressful for mice, therefore not necessarily a better model of voluntary exercise in mice (Biedermann et al. 2016).

Another limitation of the project is the use of a rodent to model human pregnancies. The use of the mouse is common since both the laboratory mouse and humans have hemochorial placentas (Turco and Moffett 2019), which means that there is a more direct contact with the maternal blood, allowing for efficient exchange of nutrients, gases, and waste products (Schmidt et al. 2015). However, besides both species having hemochorial placentas, there are many differences throughout the gestation, starting with the duration of gestation that in humans takes up to 40 weeks, while in C57BL/6J mice gestation duration is ~ 19 days. Decidualization in humans initiates in the secretory phase of the menstrual cycle, while in mice, the initiation of decidualization is dependent on the attachment of the blastocyst (Ramathal et al. 2010). While attachment of the blastocyst to the uterus is facilitated by the trophectoderm in both species, in humans it is the polar trophectoderm, the trophoblast cells on the side of the inner mass cell (IMC) that give rise to the development of the placenta, whereas in mice it is the mural trophectoderm (opposite to the IMC) that give rise to trophoblast

giant cells that will further differentiate into primary giant cells that are responsible for regulating decidualization (Elmore et al. 2022). In both species implantation occurs as an invasive process in which trophoblast cells penetrate deeply into the decidualizing uterine stroma, however, they are enabled by different cell types. In mice this process is enabled by trophoblast giant cells, while in humans is initially enabled by the syncytiotrophoblast and later by cytotrophoblast cells (Hemberger, Hanna, and Dean 2020; Turco and Moffett 2019). This leads to one of the main differences in placentation, the deep invasion of trophoblast cells into the inner third of the human myometrium, characteristic only of humans and great apes, while in mice the invasion is restricted to the decidua basalis, a phenotype associated with preeclampsia or fetal growth restriction in humans (Schmidt et al. 2015; Turco and Moffett 2019).

Another difference in the human placenta compared with that of the mouse is the site of placental exchange. The villous organization of the placenta in the human is more open than the tight maternal vascular channels known as the labyrinth of rodents (Turco and Moffett 2019). The two species differ in that the placentas of mice are trichorial, made up of three trophoblast layers between the maternal and fetal blood, whereas those of humans start as multiple layers, but end as a monochorial structure with a single syncytiotrophoblast layer facing the maternal blood (Schmidt et al. 2015; Imakawa, Nakagawa, and Miyazawa 2015). Additionally, disorders relevant in advanced maternal age, such as pre-eclampsia, are not naturally found in rodents (Turco and Moffett 2019). Nonetheless, as a model of advanced maternal age, C57BL/6 mice show many similarities in pregnancy outcomes to those observed in human pregnancies of advanced maternal age (Dalton-O'Reilly et al. 2023).

Chapter 5. Conclusion

This study highlights the impact of maternal age on the reproductive decline in a mouse model. Although the positive effects of exercise in mouse and human pregnancies have been previously reported, the reproductive performance of the Aged and Aged/Exercised group was similar in both fetal development and number of viable and unviable fetuses. There was a reduction in weight of the Aged/Exercise females at the time of pregnancy due to exercise compared to the Aged females; however, the weight reduction didn't improve pregnancy outcomes.

Related to the second aim of the study, the differential expression analysis showed no significant genes whose placental expression changed with exercise in advanced maternal age and moreover, both the Aged and Aged/Exercised gene expression showed a delay in placental development such that they were more similar to that of a placenta a day earlier in development. Overall, even though further studies are needed to assess to what extent these findings in the mouse translate to humans, our data shows that an exercise treatment does not help mitigate the effects of advanced maternal age in a mouse model.

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Appendix. Supplementary Tables and Figures

Table A1. Average per treatment group of fetal parameters by sex.

	Sex	Fetus (g)	Placenta(g)	CR Length	Width of Head	Area	Eyespot Pigmentation	Presence of Digits
Aged/ Exercised	XX	0.031 ± 0.01	0.109 ± 0.02	5.44 ± 0.71	2.77 ± 0.36	17.25 ± 4.23	1.06 ± 1.17	0.027 ± 0.10
	XY	0.041 ± 0.041	0.132 ± 0.05	6.24 ± 0.94	3.21 ± 0.48	22.25 ± 615	2.19 ± 1.31	0.349 ± 0.58
Aged	XX	0.038 ± 0.01	0.118 ± 0.02	6.09 ± 0.58	3.14 ± 0.37	20.98 ± 3.75	2.14 ± 1.31	0.043 ± 0.16
	XY	0.039 ± 0.01	0.129 ± 0.03	5.88 ± 0.74	3.13 ± 0.39	19.80 ± 5.67	1.85 ± 1.39	0.190 ± 0.43
Young	XX	0.047 ± 0.012	0.115 ± 0.02	6.45 ± 0.75	3.37 ± 0.34	23.38 ± 4.58	2.75 ± 1.02	0.470 ± 0.60
	XY	0.05 ± 0.01	0.122 ± 0.02	6.73 ± 0.61	3.42 ± 0.34	24.66 ± 4.16	2.85 ± 1.06	0.405 ± 0.59

Table A2. Average of the 3 pre matting Wheel Exercise individual measurements and the 4th measurement which was at GD10.5 of pregnanc during the dark hours.

Avg of 3 pre-matting measurement (km)	GD10.5 measurement (km)
5.7960	
4.872	
5.4517	
3.450	1.658
6.1627	0.9180
3.286	0.352
6.1987	1.2480
5.198	2.514
5.4490	0.5220
3.054	4.769
6.0843	5.4700
5.328	2.194
5.3260	2.7610
5.631	3.158
4.7263	3.5240
4.561	1.247
6.6370	1.7490
3.731	1.857
4.2470	1.5800

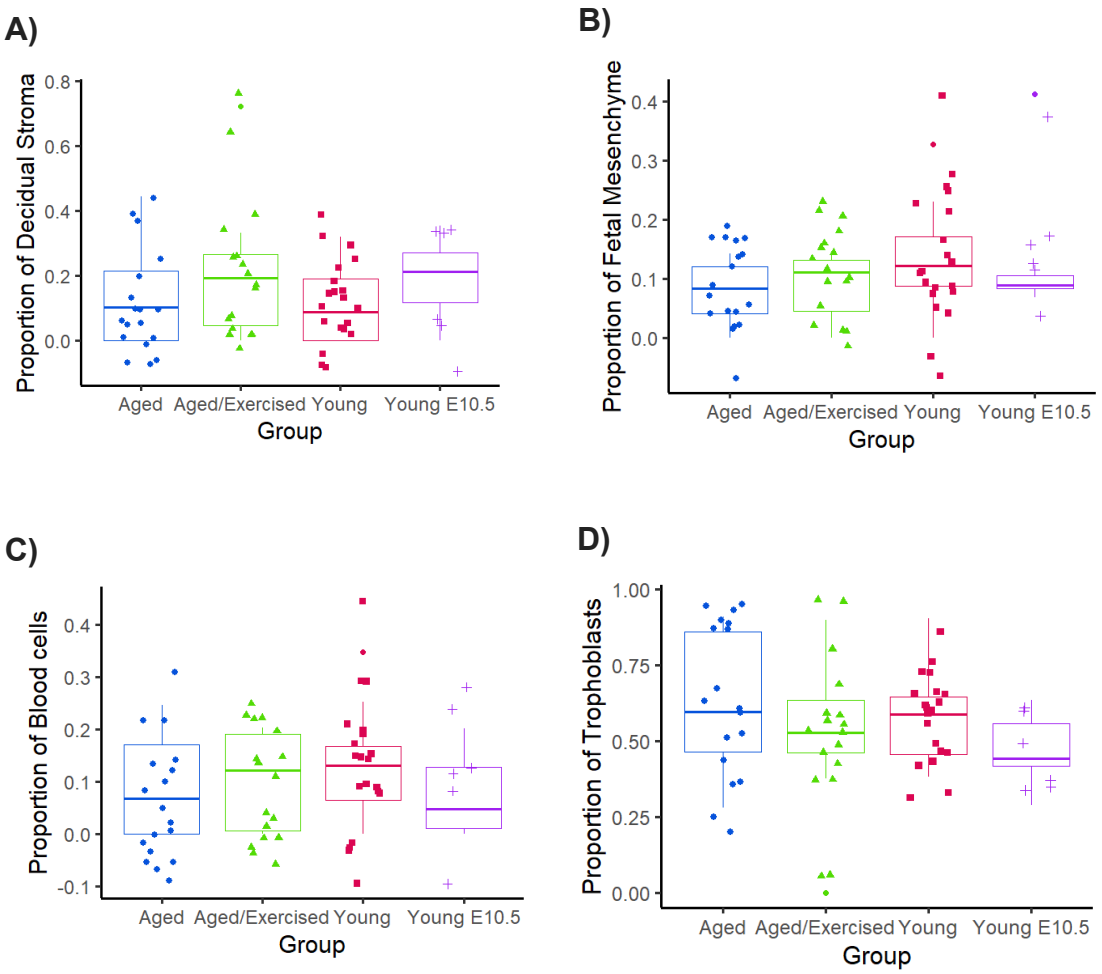

Figure A1. **Virtual deconvolution of placental RNA-seq data for cell type-specific composition. Non- statistically significant cell type differences from the virtual deconvolution of placental RNA-seq based on ANOVA from AllNuclei deconvolution: A) Decidual Stroma, B) Fetal Mesenchyme, C) Blood cells and D) Trophoblasts.**