Peri-Ovulatory Supplementation of L-Ornithine to Increase Reproductive Success in Aged Mice

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Abstract

In all mammalian species examined thus far, the ovaries produce a burst of ornithine decarboxylase (ODC) and putrescine during ovulation or after application of a bolus of human chorionic gonadotropin (hCG). Aged mice are deficient in this peri-ovulatory ODC and putrescine burst. Moreover, peri-ovulatory putrescine supplementation in aged mice increases egg quality and reduces miscarriage rates. These studies suggest that peri-ovulatory putrescine supplementation may be a simple and effective therapy for reproductive aging for women. However, putrescine has never been used in humans and, currently no pure source of putrescine is suitable for human trials. Given that ODC is highly expressed in the ovaries during ovulation but otherwise exhibits low activity in most tissues, we hypothesized that L-ornithine, the substrate of ODC, might be a better alternative. In this study, we have demonstrated that systemic application of L-ornithine increased ovarian putrescine levels; the increase was restricted to animals that had been injected with hCG. Furthermore, L-ornithine specifically increased ovarian putrescine levels without affecting putrescine levels in most other tissues. Unfortunately, thus far peri-ovulatory L-ornithine supplementation in mouse drinking water produced mixed effects on reproductive outcome in aged mice. Therefore, our studies demonstrated the potential of L-ornithine supplementation as a possible therapy for aging-related infertility, but further work is required to produce an effective application method.

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List of Abbreviations

ARG: Arginase

ARTs: Assistive Reproductive Technologies

AZ: Antizyme

AZI: Antizyme Inhibitor

cAMP: Cyclic Adenosine Monophosphate

cGMP: Cyclic Guanosine Monophosphate

dcSAM: Decarboxylated S-Adenosylmethionine

DFMO: α-Difluoromethylornithine

dpc: Days Post Coitum

eCG: Equine Chorionic Gonadotropin

EGF: Epidermal Growth Factor

eIF5A: Eukaryotic Initiation Factor 5A

GA: Gyrate Atrophy of the Choroid and Retina

GABA: γ-Aminobutyric Acid

GSA: Glutamyl-5-Semi-Aldehyde

Gy: Gyro

hCG: Human Chorionic Gonadotropin

HEK: Human Embryonic Kidney Cells

IP: Intraperitoneal

IVF: *In Vitro* Fertilization

IVM: *In Vitro* Maturation

LH: Luteinizing Hormone

MAPK: Mitogen Activated Protein Kinase

MSA: Methanesulfonic Acid

NPPC: Natriuretic Peptide Precursor Type C

NPR2: Natriuretic Peptide Receptor 2

OAT: Ornithine Aminotransferase

ODC: L-ornithine Decarboxylase

ORNT1: Ornithine Transporter 1

OTC: Ornithine Transcarbamylase

P5C: Pyrroline-5-Carboxylate

PAO: Acetylpolyamine Oxidase

PBS: Phosphate Buffered Saline

PDE3A: Phosphodiesterase 3A

PGCs: Primordial Germ Cells

ROS: Reactive Oxygen Species

SAM: S-Adenosylmethionine

SAM-DC: S-Adenosylmethionine Decarboxylase

SMOX: Spermine Oxidase

SMS: Spermine Synthase

SRM: Spermidine Synthase

SRS: Snyder-Robinson Syndrome

SSAT: Spermidine/Spermine N^1 Acetyl Transferase

TCA: Trichloroacetic Acid

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1. Introduction

1.1. Reproductive Aging and Infertility

An increasing tendency in modern industrialized society is to delay childbearing until later in life. However, this delay has a large impact on fertility. According to *Statistics Canada*, the percentage of women having children at the age of 35 years and beyond has nearly doubled from 10.8% in 1993 to 20% in 2013. In the same period, the percentage of women waiting until at least age 35 to have their first child has doubled from 6.1% in 1993 to 12.9% in 2013 (https://www.statcan.gc.ca/pub/82-625-x/2016001/article/14673-eng.htm). Attempting to conceive a child after 35 years of maternal age is significantly more difficult compared to younger ages. Women are delaying childbearing in many developed countries including the US, England, and France (Baird et al., 2005; Maher and Macfarlane, 2004) due to economic, social, and technological changes (Huang et al., 2008). Delayed childbearing is accompanied by reduced oocyte quality (Navot et al., 1991; Wang et al., 2012), which increases aneuploid conception (Benadiva et al., 1996; Demko et al., 2016) and the risk of miscarriage (Haadsma et al., 2010; Nybo Andersen et al., 2000).

Compared to human males, who produce sperm from germline stem cells throughout adulthood, females have a limited supply of oocytes for their reproductive lifespan. Oocyte formation begins during gastrulation in fetal development. During gastrulation, uncommitted epiblast cells differentiate and commit to becoming primordial germ cells (PGCs). The PGCs then migrate to the area of the prospective gonads where they differentiate further to become oogonia. These oogonia then begin to proliferate and form groups, known as germ cell clusters. Within these clusters, the oogonia undergo further DNA replication, entering meiosis where they are known as primordial oocytes. Meiosis only progresses through the

leptotene, zygotene, and pachytene stages of prophase I, where it arrests at the diplotene stage of prophase I (van den Hurk and Zhao, 2005). The process of oogenesis endows women with the total number of possible oocytes for their reproductive lifespan, which is also termed the ovarian reserve (Hansen et al., 2012). At birth, it has been estimated that women have approximately 295,000 non-growing follicles. Unfortunately, with age this number decreases such that there is an estimated 180,000 non-growing follicles at age 13, 65,000 at age 25, 16,000 at age 35, and less than 1,000 by the age of menopause (Wallace and Kelsey, 2010). Thus, increased maternal age is not only associated with decreased oocyte quality (Navot et al., 1991; Wang et al., 2012), but also with decreased numbers of oocytes.

Due to the reproductive challenges associated with advanced maternal age, many women seek help from assistive reproductive technologies (ARTs). ARTs such as *in vitro* fertilization (IVF) are powerful technologies to assist conception but they also have some disadvantages. Women undergoing IVF treatments utilizing their own eggs exhibit age-dependent increases in spontaneous abortion rates comparable to the general population (Haadsma et al., 2010; Schieve et al., 2003). However, patients utilizing donor oocytes, generally from young donors (Sauer and Kavic, 2006), have relatively low spontaneous abortion rates irrespective of age (Schieve et al., 2003). Taken together, these studies support the notion that poor oocyte quality is of the utmost importance in reproductive aging.

1.2. Mammalian Oocyte Prophase Arrest

As previously mentioned, primordial oocytes remain arrested at the diplotene stage of prophase I throughout follicular development and growth, which can last as long as months in mice, or decades in humans (McGee and Hsueh, 2000).

In the ovaries, oocytes are maintained at the diplotene stage of prophase I by intricate signalling pathways involving both cellular (Zhang et al., 2010) and molecular interactions (Jones, 2008). High levels of cyclic adenosine monophosphate (cAMP) in the oocytes ensure prophase I arrest is maintained (Jones, 2008). cAMP is produced in the oocyte by a constitutively active heterotrimeric G protein (G_S)-linked receptor, GPR_3/GPR_{12}. This G protein complex stimulates adenylyl cyclase to synthesize cAMP in the oocyte (Hinckley et al., 2005; Mehlmann, 2005; Mehlmann et al., 2002).

The oocyte is further surrounded by layers of somatic cells. The first few layers of cells directly surrounding and in contact with the oocyte are cumulus cells. Some but not all the cumulus cells enclosing the oocyte are attached to other somatic cells known as mural granulosa cells. Both cumulus and mural granulosa cells play roles in regulating cAMP concentrations in the oocyte. Mural granulosa cells secrete natriuretic peptide precursor type C (NPPC). NPPC then binds and activates natriuretic peptide receptor 2 (NPR2), a guanylyl cyclase, on cumulus cells surrounding the oocyte creating cyclic guanosine monophosphate (cGMP). The cGMP created by NPR2 then diffuses through gap junctions, composed of the protein connexin 43, from the cumulus cells into the oocyte. cGMP then inhibits the cAMP degrading enzyme phosphodiesterase 3A (PDE3A) in the oocyte (Zhang et al., 2010). Together, the combined action of cAMP production and PDE3A inhibition by cGMP ensures high levels of cAMP and meiotic arrest at prophase I.

1.3. Mammalian Ovulation

As oocytes are maintained at prophase I of meiosis, a hormonal trigger causes the resumption of meiosis shortly before ovulation after the onset of puberty (Pelosi et al., 2015). Prior to ovulation, luteinizing hormone (LH) binds to the LH receptor on mural granulosa

cells (Amsterdam et al., 1975; Peng et al., 1991). The same LH receptor is also activated by the ovulatory hormone human chorionic gonadotropin (hCG). Binding of the LH receptor promotes the production of epidermal growth factor (EGF) like proteins within mural granulosa cells (Park et al., 2004). EGFs rapidly inhibit NPR2 activity (Egbert et al., 2014; Liu et al., 2014; Robinson et al., 2012) and activate mitogen-activated protein kinase (MAPK) to phosphorylate connexin 43 connecting the cumulus cells and the oocyte (Norris et al., 2008; Norris et al., 2009; Sela-Abramovich et al., 2005; Sela-Abramovich et al., 2006). Inhibition of NPR2 prevents cGMP production in the cumulus cells. Phosphorylation of connexin 43 reduces gap junction permeability thereby reducing cGMP secretion to the oocyte (Norris et al., 2008; Norris et al., 2009). There is also a slow decrease in NPPC secretion by mural granulosa cells reducing activation of NPR2 (Egbert et al., 2014; Liu et al., 2014; Robinson et al., 2012). These two processes result in a net decrease of cGMP in the oocyte. This allows PDE3A to hydrolyze cAMP in the oocyte, promoting the resumption and continuation of meiosis.

1.4. Oocyte Maturation and Quality

Binding of LH to the LH receptor re-initiates meiosis in the oocyte, indirectly as described above. This allows the oocyte to complete meiosis I where the oocyte eliminates one set of homologous chromosomes in polar body I. The oocyte then continues into meiosis II, such that by the time the oocyte is ovulated, it re-arrests at metaphase II. Meiosis only proceeds to the end if/when the oocyte is fertilized by sperm where it eliminates one set of sister chromatids in polar body II. The period of time from meiotic re-entry to metaphase II re-arrest is known as oocyte maturation (Jamnongjit and Hammes, 2005). Oocyte maturation occurs concurrently with ovulation and can be subdivided into nuclear and cytoplasmic

maturation (Eppig et al., 1994). Nuclear maturation refers to the active progression of meiosis in the oocyte where it eliminates one set of homologous chromosomes in polar body I and re-arrests at metaphase II awaiting fertilization. Cytoplasmic maturation includes a variety of processes including the relocation of organelles, synthesis of oocyte-specific proteins, and alterations of membrane transport systems (Moor et al., 1998). Both nuclear and cytoplasmic maturation must occur synchronously in order for the oocyte to be endowed full developmental competence (Moor et al., 1998). When nuclear maturation occurs in the absence of cytoplasmic maturation, the oocyte is unable to support fertilization and development of the embryo to term (Eppig et al., 1994). Therefore, to ensure a high-quality oocyte capable of supporting fertilization and successful embryogenesis both of these programs must occur together.

Unfortunately, as maternal age increases many of the processes involved in nuclear and cytoplasmic maturation become flawed or occur improperly. This leads to drastic effects on oocyte quality that are generally detrimental to reproductive potential. Issues affecting oocyte nuclear and cytoplasmic maturation with increased maternal age are numerous, some examples are provided. Increased maternal age is associated with increased incidence of incorrect chromosomal separation. This creates an oocyte with an incorrect number of chromosomes known as aneuploidy, many of which are not survivable (Chiang et al., 2010; Duncan et al., 2012; Kuliev et al., 2011). In addition, increased maternal age has been associated with decreases in mitochondrial number and altered mitochondrial distribution and activity (Liu et al., 2016b; Udagawa et al., 2014; Wai et al., 2010). These decreases and alterations can detrimentally affect mitochondrial dynamics or energetics in the oocyte leading to issues such as insufficient ATP production causing issues during embryonic

development. Last, during oocyte maturation, chromatin-associated histones are generally fully deacetylated and the chromatin is transcriptionally silent (Akiyama et al., 2004; Akiyama et al., 2006). However, increases in maternal age have been associated with insufficient levels of histone deacetylation. This can cause insufficient chromatin compaction, which can lead to issues with chromosome separation and can cause aneuploidy (Gu et al., 2010). Together, all these issues may result in poorer oocyte quality and diminished developmental potential of oocytes.

Given the critical role of oocyte maturation in egg quality, improving the oocyte maturation process in aged animals may improve egg quality. In this regard, supplementation of the polyamine putrescine during oocyte maturation/ovulation might be a useful strategy to lessen the deterioration in oocyte quality observed with increased maternal age.

1.5. Polyamines

The polyamines putrescine, spermidine, and spermine are ubiquitous poly-cationic molecules found in all cells that play important biological roles. Some of the defined roles *in vivo* include: the maturation of eukaryotic initiation factor 5A (eIF5A) (Nishimura et al., 2012; Pallmann et al., 2015; Sievert et al., 2014), regulation of ion channels (Pegg, 2009, 2016), roles in heritable disease (Cason et al., 2003), and roles in reproduction (Liu et al., 2016a; Liu and Tao, 2012; Tao et al., 2015).

1.5.1. Polyamine Biosynthesis

The polyamine biosynthetic pathway begins with the enzyme L-ornithine decarboxylase (ODC). ODC is the first and rate-limiting reaction in polyamine biosynthesis. ODC catalyzes the decarboxylation of L-ornithine to produce putrescine (a diamine).

Putrescine is the precursor to the higher polyamines spermidine (a triamine) and spermine (a tetraamine). To produce spermidine and spermine, S-adenosylmethionine (SAM) becomes decarboxylated by the action of SAM decarboxylase (SAM-DC) producing decarboxylated SAM (dcSAM). The enzyme spermidine synthase then transfers an aminopropyl moiety from dcSAM to putrescine to produce spermidine. In a similar manner, spermine synthase transfers an aminopropyl moiety from dcSAM to spermidine to produce spermine (figure 1).

The polyamines can also be interconverted. This occurs through two enzymatic steps involving sequential action of spermidine/spermine N^1 acetyl transferase (SSAT) and acetylpolyamine oxidase (PAO). SSAT can monoacetylate spermidine or mono- or di-acetylate spermine. Acetylated polyamines have two fates: export via a transport protein and elimination in urine (Gerner and Meyskens, 2004) or conversion back to lower polyamines by PAO. In addition to the action of SSAT and PAO to convert spermine to spermidine, spermine can be directly converted to spermidine via the action of spermine oxidase (SMOX) (Pegg, 2016) (figure 1). The importance of the SSAT-mediated back conversion pathway is unknown. Embryonic knockout of SSAT in mice was not observed to affect life expectancy, tissue polyamine pools, or cause phenotypic abnormalities when compared to controls (Niiranen et al., 2006). Polyamines are available through diet (Atiya Ali et al., 2011; Bardocz et al., 1995; Nishibori et al., 2007; Okamoto et al., 1997) and are produced by gut bacteria where they can be absorbed (Kibe et al., 2014; Noack et al., 2000; Sugiyama et al., 2017). Given that the transport proteins responsible for polyamine translocation remain poorly characterized in mammals (Pegg and Casero, 2011), the complete method of polyamine uptake and efflux is not well understood.

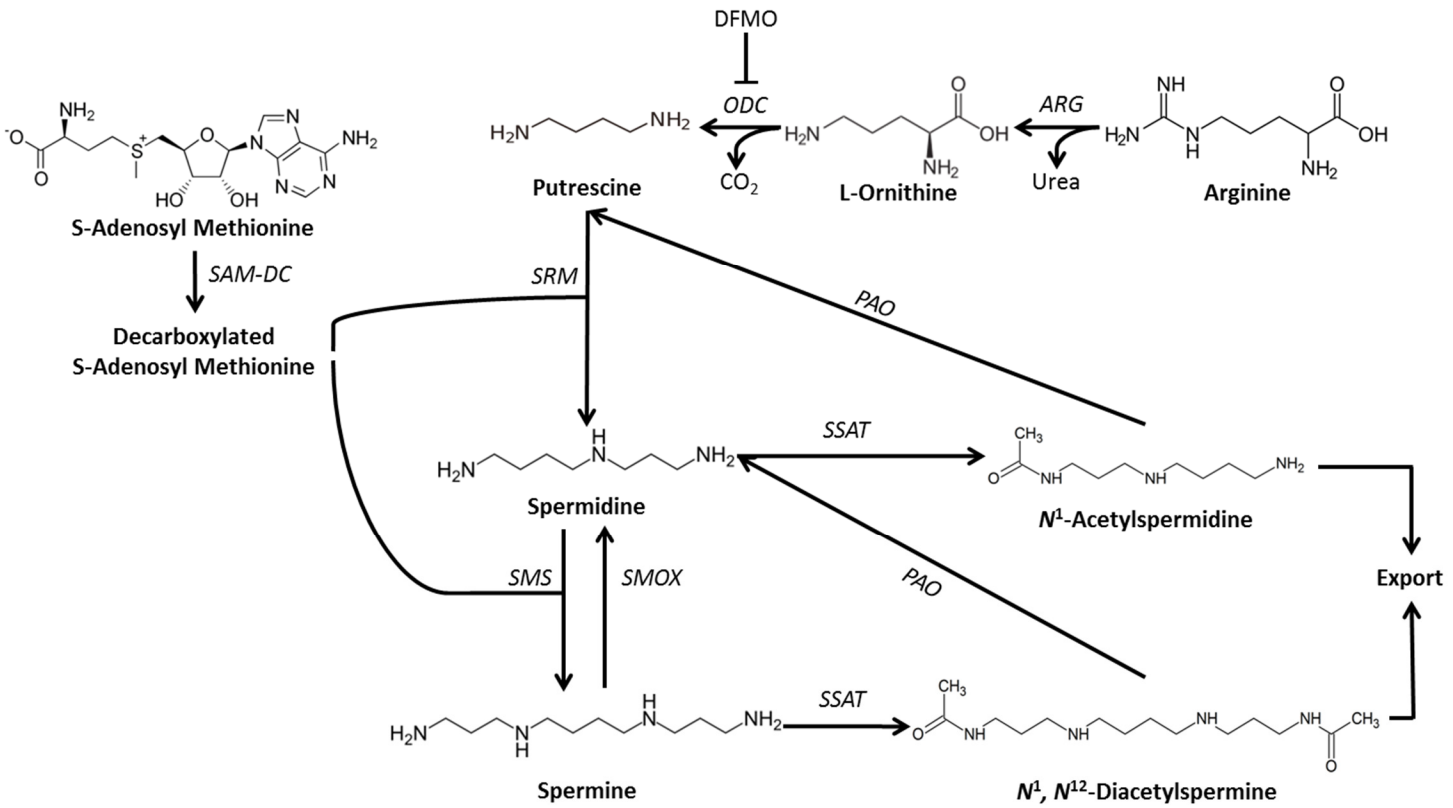

Figure 1: Polyamine structures, synthesis, and inter-conversion. Enzymes are identified in italics. Arginase (ARG) converts arginine into L-ornithine. Ornithine decarboxylase (ODC) then catalyzes the reaction of L-ornithine into putrescine and can be irreversibly inhibited by α-difluoromethylornithine (DFMO). In order to create the higher polyamines spermidine and spermine, S-adenosyl methionine (SAM) becomes decarboxylated by SAM-decarboxylase (SAM-DC) to form decarboxylated SAM. Spermidine is produced by the action of spermidine synthase (SRM) from putrescine and an amino propyl group from decarboxylated SAM. Likewise, spermine is produced by spermine synthase (SMS) from spermidine and an amino propyl group from decarboxylated SAM. The polyamines can be reversely converted. Spermine can be directly converted to spermidine by spermine oxidase (SMOX). Spermidine/spermine N^1 acetyl transferase (SSAT) monoacetylates spermidine and can mono- or di-acetylate spermidine. These acetylated polyamines are then either exported or converted to the lower polyamines by acetylpolyamine oxidase (PAO).

1.5.2. Ornithine Decarboxylase (ODC)

ODC is the rate-limiting reaction in the production of polyamines. Active ODC is a homodimer with two active sites consisting of residues from both monomers (Coleman et al., 1994). Eukaryotic ODC is highly specific for L-ornithine and demonstrates 250-fold lower activity for L-lysine and 1,000-fold lower activity for L-arginine compared to L-ornithine (Osterman et al., 1995). The association between ODC dimers is unusually weak. Thus, under physiological conditions active ODC dimers and inactive ODC monomers are in rapid equilibrium (Pegg, 2006).

In vitro assays comparing the enzymatic activity of ODC and spermidine and spermine synthases in tissue extracts have been undertaken (Raina et al., 1976). They revealed that ODC activity is approximately 10- to 100-fold lower compared to the activity of spermidine or spermine synthases (Raina et al., 1976). In addition, ODC has a very short half-life, measuring approximately 10-20 minutes *in* vivo (Iwami et al., 1990; Russell and Snyder, 1969; Tabor and Tabor, 1984). Since ODC activity is low in most tissues, concentrations of putrescine are generally low compared to spermidine and spermine (Nishimura et al., 2006; Wagner et al., 1984). The difference in putrescine concentrations was further demonstrated in studies examining the putrescine concentrations in regenerating rat liver. The half-life of putrescine was shown to only be approximately two hours compared to the roughly four day half-life of spermidine (Russell et al., 1970). These findings further explain why only low levels of putrescine are observed in most tissues compared to spermidine and spermine.

Regulation of ODC

ODC is regulated in a complex manner, but is predominantly controlled at the level of protein longevity (Pegg, 2006). ODC monomers are targeted for proteasomal degradation in cells in a ubiquitin independent manner (Murakami et al., 1992). Antizyme (AZ) binds to ODC monomers with high affinity. This prevents ODC monomers from associating into an active dimer form. Once bound, AZ directs the ODC monomer to the 26S proteasome for degradation. However, AZ is not degraded by the proteasome allowing AZ to target other ODC monomers for degradation (Coffino, 2001; Murakami et al., 1992).

Levels of AZ are regulated by polyamine concentrations. AZ expression is induced by high intracellular concentrations of polyamines and suppressed by low concentrations (Petros et al., 2005). AZ also inhibits import of extracellular polyamines and increases the rate of export of acetylated polyamines (Mitchell et al., 1994; Sakata et al., 1997; Sakata et al., 2000; Suzuki et al., 1994).

In humans, there are three AZ homologues (Ivanov and Atkins, 2007; Ivanov et al., 2000). AZ1 and AZ2 are expressed in most tissues except for haploid germ cells in the testes. AZ2 is expressed at much lower levels compared to AZ1 and does not degrade ODC in *in vitro* assays, but inhibits polyamine import (Zhu et al., 1999). AZ3 expression is restricted to the testes and regulates ODC activity during spermatogenesis in haploid germ cells (Tosaka et al., 2000).

All members of the AZ family are regulated by AZ inhibitor (AZI). AZI inactivates all members of the AZ family (Mangold and Leberer, 2005) preventing ODC degradation. AZ has a higher affinity for AZI than it does for ODC. Thus, AZI can displace bound ODC

monomers from AZ. This allows the ODC monomers to dimerize and become catalytically active (Pegg, 2006; Perez-Leal and Merali, 2012).

Importance of ODC

Polyamines play an important role in cell proliferation from yeast (Schwartz et al., 1995) to mammals (Pendeville et al., 2001). Loss of ODC expression in haploid yeast induced by loss of function mutations results in growth arrest (Schwartz et al., 1995). Upon supplementation of exogenous putrescine, spermidine, and spermine, growth resumes and the yeast appear phenotypically normal (Schwartz et al., 1995). Furthermore, when *ODC* is knocked out embryonically in mice, the embryos die *in utero* between embryonic days four and six (E4 to E6) (Pendeville et al., 2001). Some level of proliferation occurs prior to embryonic death as these embryos persist through the blastocyst stage. However this is thought to be largely attributed to maternally derived ODC in the oocyte (Pendeville et al., 2001).

Consistent with the role of ODC in cell proliferation, ODC and SAM-DC activity increase sharply from E6 to E8 during embryogenesis in mice, a time of high cellular proliferation (Fozard et al., 1980a). The role of ODC during embryogenesis has been studied with α-difluoromethylornithine (DFMO), a potent ODC inhibitor. Treatment of pregnant mice with DFMO in drinking water from E5 to E8 caused embryonic death such that no evidence of pregnancy was observed at E18 (Fozard et al., 1980a). This DFMO treatment was observed to abolish increases in uterine ODC activity as well as concentrations of putrescine, and spermidine. DFMO treatment was observed to have the same effect on embryogenesis in both rats and rabbits (Fozard et al., 1980b). These studies identified ODC activity as essential for cell proliferation and thus for embryogenesis.

As ODC is important for proper cell growth and proliferation it is unsurprising that it is up-regulated in various cancers (Gerner and Meyskens, 2004). However, in most cancers increased ODC activity is usually accompanied by increased activity of SAM-DC (Russell and Levy, 1971). The increase in the enzymatic activity of ODC and SAM-DC results in increased levels of not only putrescine, but spermidine and spermine as well (Andersson and Heby, 1972). Thus, the rapid proliferation of cancer cells may be due to increases in spermidine and spermine concentrations in addition to putrescine concentrations.

Despite the correlation of increased ODC activity and cancers, increases in putrescine alone have not been observed to increase cellular proliferation rates (Wilson et al., 2005). Transgenic human embryonic kidney (HEK) cells transfected with a doxycycline inducible ODC have approximately 50-fold higher ODC activity and 9-fold higher putrescine concentrations when treated with doxycycline compared to controls (Wilson et al., 2005). No differences in spermidine and spermine concentrations were observed in transgenic doxycycline exposed cells compared to control cells. Despite the difference in putrescine concentrations, no difference in the rate of cell proliferation was observed compared to controls (Wilson et al., 2005).

Similarly, transgenic ODC overexpressing mice which demonstrate 20- to 50-fold more ODC activity in virtually all tissues have been examined. Interestingly, they exhibit similar levels of spermidine and spermine compared to wild type mice (Halmekytö et al., 1993). However, they have extremely elevated putrescine concentrations in almost all tissues (Halmekytö et al., 1993; Halmekyto et al., 1991). Despite increased putrescine concentrations, these transgenic mice show no phenotypic differences or differences in tumor rates compared to wild type mice, even up to two years of age (Alhonen et al., 1995). Thus,

it appears that for putrescine to increase cell proliferation it must be converted to spermidine or spermine via SAM-DC activity.

1.5.3. Polyamine Roles

Polyamines play specific and different roles *in vivo*. Although some polyamine roles have yet to be elucidated, some of the better-defined roles of the individual polyamines are provided.

Spermidine

The best-defined role of spermidine is in the post-translational maturation of eukaryotic initiation factor 5A (eIF5A) (Park, 2006; Park et al., 2010; Pegg and Casero, 2011). eIF5A plays a role in translational initiation (Gregio et al., 2009) and is implicated in the synthesis of proteins containing multiple consecutive proline residues (Doerfel et al., 2013; Gutierrez et al., 2013). Currently, eIF5A is the only known protein to contain the amino acid hypusine (N^{ε}-(4-amino-2-hydroxybutyl) lysine) (Park, 2006). Spermidine is utilized to convert lysine 50 of human eIF5A into hypusine (Park, 2006). This process is completed in two enzymatic steps. First, deoxyhypusine synthase transfers a 4-aminobutyl moiety from spermidine to the ε-amino group of lysine. Second, deoxyhypusine hydroxylase converts the intermediate to produce hypusine (Park, 2006; Park et al., 2010) (appendix, figure 11).

This amino acid conversion is essential in mice. Embryonic knockout of either *eif5a* itself (Nishimura et al., 2012), deoxyhypusine synthase, or deoxyhypusine hydroxylase causes embryonic death early in embryogenesis (Pallmann et al., 2015; Sievert et al., 2014).

Spermine

Spermine has been shown to have several functions *in vivo.* These functions include roles in neurological activity through the regulation of ion channels, including potassium, glutamate, and calcium channels (Pegg, 2009, 2016). The best described role for spermine is in neurological function, which is supported by studies in Gyro (Gy) mice. The Gy mouse model was originally generated by x-irradiation and has retained mutations which make it ideal for studying defects in the polyamine pathway. Male Gy mice exhibit hypophosphatemia with rickets, sterility, deafness, reduced size and viability, and neurological abnormalities (Lyon et al., 1986). The abnormalities observed in the male Gy mice are a result of the deletion of the 5' end of a phosphate-regulating gene named *Phex*, and of the spermine synthase gene (Lorenz et al., 1998; Meyer et al., 1998). As a result of the deletion of the spermine synthase gene, there is very little spermine in the tissues of Gy mice (Lorenz et al., 1998; Meyer et al., 1998). The reduced size and viability, sterility, deafness, and neurological abnormalities were determined to be caused specifically by the loss of the spermine synthase gene. This was confirmed by examining Hyp mice, which only lack the *Phex* gene. Hyp mice do not exhibit phenotypes other than hypophosphatemia and rickets (Lorenz et al., 1998; Meyer et al., 1998). Thus, the additional phenotypes exhibited in Gy mice are due to the loss of spermine synthase activity, or the loss of both *Phex* and spermine synthase activity. Together, these findings indicate that spermine is essential for neurological activity, growth, and fertility in male mice (Pegg, 2016).

In humans, alterations in spermine synthase cause Snyder-Robinson syndrome (SRS), an X-linked developmental disease including intellectual disability (Cason et al., 2003). SRS has a similar phenotype to Gy mice, albeit less severe. As SRS is an X-linked disorder, it affects all males with an affected X-chromosome and only females with two affected X-

chromosomes. SRS causes intellectual disability, speech abnormalities, muscle hypoplasia, diminished body bulk, hypotonia, and some form of osteoporosis (Pegg, 2016). To date, SRS is the only known polyamine deficiency syndrome in humans (Schwartz et al., 2011).

Putrescine

The roles of putrescine, other than being the precursor for the other polyamines, remain poorly characterized compared to the roles of spermidine and spermine. As mentioned previously, tissue concentrations of putrescine are generally very low compared to concentrations of spermidine and spermine (Nishimura et al., 2006). This is due to the short half-life of both ODC and putrescine (Russell et al., 1970; Russell and Snyder, 1969). ODC expression can be triggered by many physiological triggers including hormones, drugs, tissue regeneration, and growth factors (Maudsley and Kobayashi, 1974; Pegg, 2016; Pegg and McCann, 1982). Increases in ODC expression then control putrescine concentrations.

In the brain, putrescine has been observed to be converted into the neurotransmitter γ-aminobutyric acid (GABA). This occurs by N-acetylation of putrescine followed by the action of monoamine oxidase (Seiler and Al-Therib, 1974). This conversion may be related to a neuroprotective role of putrescine, but not spermidine or spermine on seizure susceptibility that has been observed in *Xenopus laevis* tadpoles (Bell et al., 2011). More recently, it has been discovered that putrescine may play a vital role in reproductive success.

1.6. Putrescine in Reproduction

It has been known for decades that a surge of LH on the evening of proestrus causes a large increase in ovarian ODC activity in rats (Kaye et al., 1973; Kobayashi et al., 1971). During this time, ovarian ODC activity is 10-fold higher compared to any other time in the

four day estrus cycle (Kobayashi et al., 1971). The increase in ODC activity requires active transcription and translation of the ODC gene and mRNA, respectively (Kaye et al., 1973; Maudsley and Kobayashi, 1974). As opposed to embryogenesis where activities of both ODC and SAM-DC increase (Fozard et al., 1980b), only ODC activity is increased during the estrus cycle. Thus, only increases in ovarian putrescine concentrations occur (Tao et al., 2015).

In addition to rats, other rodents such as mice (Tao and Liu, 2013) and hamsters (Persson et al., 1986) exhibit LH/hCG-mediated increases of ODC activity. In mice, peak increases of putrescine occur approximately five hours after hCG administration and then slowly decline (Tao et al., 2015). The increase in ODC activity in the ovary is localized to antral follicles (Icekson et al., 1974), specifically in the mural granulosa cells, cumulus cells, and the oocyte itself (Bastida et al., 2005; Tao and Liu, 2013).

In mice, the transient increase in ODC and putrescine concentrations take place concurrently with oocyte maturation (Eppig et al., 1994). Supplementation of drinking water with the ODC inhibitor DFMO during ovulation inhibits increases in ovarian putrescine (Bastida et al., 2005; Tao and Liu, 2013) without affecting ovarian spermidine or spermine concentrations (Tao et al., 2015). Similar to studies in rats supplemented with DFMO (Fozard et al., 1980c), DFMO supplementation in mice did not affect the number of ovulated oocytes (Tao and Liu, 2013). However, DFMO supplemented to mice in drinking water during ovulation significantly increased the rate of chromosomal aneuploidies in oocytes from 1.3% in control animals to 7.7% in DFMO treated animals (Tao and Liu, 2013).

Old mice exhibit age-associated declines in ODC activity during the peri-ovulatory period. In aged mice, decreased ODC activity only affects ovarian putrescine concentrations, not spermidine or spermine concentrations (Tao et al., 2015). Consistent with the observation of DFMO-mediated ODC inhibition in young mice, old mice exhibit reduced ODC activity and correspondingly increased rates of oocyte aneuploidy (Tao and Liu, 2013). When drinking water was supplemented with putrescine, immature oocytes were harvested, and then matured *in vitro* in the presence of putrescine, the rates of oocyte aneuploidy significantly decreased from 12.7% in untreated control oocytes to 5.3% in treated oocytes (Tao and Liu, 2013). Furthermore, supplementing in *in vitro* maturation media with 0.5 mM of putrescine, corresponding to approximately peak ovarian concentrations during ovulation in young mice, increased the quality of blastocysts produced from aged oocytes (Liu et al., 2016a). Together, these results suggest that putrescine is an important factor in the prevention of aneuploidy and is important for oocyte maturation and proper chromosome segregation.

To investigate the effect of putrescine supplementation on reproductive outcome in old animals, putrescine was supplemented in drinking water (1% w/v) during mating. Peri-ovulatory putrescine supplementation showed positive results. Putrescine supplementation in old animals was capable of not only restoring ovarian putrescine concentrations, but also increased blastocyst cell numbers, reduced embryonic resorption rates, and doubled the number of live births (Tao et al., 2015). Moreover, supplemental putrescine showed no toxicity to mothers or fetuses when supplemented outside the peri-ovulatory timeframe. The progeny produced from putrescine supplementation were phenotypically and reproductively normal. Supplemental putrescine was easily excreted and had a residence time of approximately one hour in blood and two hours in the ovary (Tao et al., 2015).

Thus, peri-ovulatory putrescine supplementation may be a practical, simple, and effective therapeutic solution to rescue oocyte quality and reproductive outcome with aging. However, even though putrescine is relatively non-toxic (LD_{50} 2000 mg/kg (Til et al., 1997)) and unequivocally rescues reproductive capabilities in old mice, its application in humans is hindered. Unfortunately, there is no putrescine supplement available for use in humans, preventing the testing and application of putrescine. In addition, as the exact mechanism of action of putrescine remains unknown, it may be preferable to avoid systemic increases in putrescine concentrations.

1.7. L-Ornithine

We decided to explore the possibility of utilizing L-ornithine as an additional or alternative treatment to improve reproductive potential in old mice. L-ornithine is an amino acid that is not incorporated into proteins (Hayasaka et al., 2011). L-ornithine is synthesized by the enzyme arginase using arginine as a substrate, with urea as a by-product (figure 1) (Yu et al., 2003). It is also readily absorbed in the gut (Sakamoto et al., 2015) and is available from a variety of dietary sources (Ishida et al., 2013).

L-ornithine is found at varying concentrations in many tissues including the liver, eyes, blood, and brain (Daune et al., 1988; Seiler et al., 1989; Takach et al., 2014). In the body, arginase is widely expressed (Yu et al., 2003). Due to this widespread expression, it is thought that arginase may synthesize L-ornithine for metabolic processes in some tissues. Interestingly, arginase activity is relatively low in tissues that have extensive blood circulation such as the liver, bone marrow, and spleen (Yu et al., 2003). This may indicate these tissues can obtain L-ornithine that is required from the circulation where it is readily available in plasma (Pegg and McCann, 1982). However, arginase activity was readily

detected in highly regenerative tissues with limited blood supply such as the surface epithelium of the esophagus, lymphocytes in the lymph nodes and thymus, and the endometrium of the uterus (Yu et al., 2003). This may indicate that in tissues with limited access to blood flow, arginase activity may play a larger role in the synthesis of L-ornithine to generate the metabolic products of L-ornithine, proline, glutamate, and polyamines.

1.7.1. L-Ornithine Metabolism

There are three major metabolic pathways in which L-ornithine is involved: polyamine production through ODC, ammonia detoxification in the urea cycle through arginase and ornithine transcarbamylase (OTC), and the synthesis of glutamate and proline through ornithine aminotransferase (OAT).

L-ornithine and urea are produced from arginine by arginase as an intermediate in the urea cycle. Arginase is a cytoplasmic enzyme and the last step of the urea cycle that converts ammonia into urea (Author, 1988). L-ornithine synthesized by arginase is shuttled into the mitochondrial matrix by the mitochondrial membrane transport protein ornithine transporter 1 (ORNT1) (Camacho et al., 2006). Once in the mitochondrial matrix, it is converted into citrulline by OTC to continue the urea cycle (Author, 1988) (appendix figure 12).

There are two important pieces of information to consider regarding arginase and OTC. First, the full urea cycle only occurs in the periportal hepatocytes of the liver (Crombez and Cederbaum, 2007). However, the individual enzymes may be found at lower levels in other tissues where they may play other metabolic roles (Crombez and Cederbaum, 2007). Second, the L-ornithine produced in the liver and utilized in the urea cycle is largely recycled

(Morris, 2002). Thus, liver arginase activity is not a significant source of L-ornithine for ODC or OAT in other tissues (Morris, 2002).

OAT catalyzes the reversible conversion of L-ornithine to glutamate or proline (Lim et al., 1998) (appendix, figure 13). In mammals, OAT is a mitochondrial matrix enzyme that is expressed in most tissues with the highest activities found in the liver, kidney, intestine, and brain (Ginguay et al., 2017; Lim et al., 1998). Interestingly, the OAT reaction proceeds towards synthesis of glutamate or proline or towards synthesis of L-ornithine depending on the tissue. In the liver, kidney, and brain, the enzymatic reaction of OAT proceeds towards synthesis of glutamine and proline from L-ornithine (Levillain et al., 2000; Matsuzawa and Obara, 1987; McGivan et al., 1977; Wroblewski et al., 1985). However, in the intestine the enzymatic activity of OAT proceeds towards L-ornithine synthesis from glutamine (Tomlinson et al., 2011). The intestinal synthesis of L-ornithine via OAT is important in neonatal mice (Wang et al., 1995). Proline in maternal milk is converted into L-ornithine and subsequently arginine in the intestinal epithelium of neonatal mice (Ginguay et al., 2017). When OAT is disrupted in neonates, L-ornithine concentrations increase while arginine concentrations decrease. This results in a net accumulation of ammonia that is not converted into urea resulting in neonatal death (Wang et al., 1995).

1.7.2. L-Ornithine Supplementation as an Alternative to Putrescine

L-ornithine supplementation may offer distinct advantages over putrescine when considering its use to improve reproductive outcome in aging mammals. L-ornithine is widely available as a dietary supplement and has safety data readily available. Therefore, if L-ornithine is found to be effective, it could be more easily utilized to test its effect in humans. In contrast, putrescine has never been administered in humans, except through food

intake, and therefore its safety in humans is unknown. However, both putrescine and L-ornithine have low oral toxicities and are relatively safe; the LD_{50} for putrescine is 2,000 mg/kg (Til et al., 1997) and that of L-ornithine is 10,000 mg/kg (Breglia et al., 1973).

The largest possible advantage that using L-ornithine may provide is specific increases in ovarian putrescine concentrations. ODC activity during the peri-ovulatory period is LH/hCG-dependent (Kobayashi et al., 1971; Maudsley and Kobayashi, 1974) and is specifically elevated in the ovary (Bastida et al., 2005; Icekson et al., 1974; Tao and Liu, 2013). Thus, supplemental L-ornithine may specifically produce putrescine in the ovary where it can have beneficial effects (Liu et al., 2016a; Liu and Tao, 2012; Tao et al., 2015) while avoiding systemic increases of putrescine.

1.8. Project Hypothesis and Objectives

The overall hypothesis of this project is given that ODC is produced in the ovary during the peri-ovulatory period (Maudsley and Kobayashi, 1974), L-ornithine supplementation may specifically increase ovarian putrescine. The increase of putrescine during this period may provide reproductive benefit to old mice. We hypothesize that increases in ovarian putrescine caused by peri-ovulatory L-ornithine supplementation may provide similar reproductive benefits to those observed with peri-ovulatory putrescine supplementation (Tao et al., 2015; Tao and Liu, 2013). In order to investigate this possibility, two overall objectives will be explored:

i. To determine whether peri-ovulatory L-ornithine supplementation specifically increases putrescine in the ovaries in mice.

ii. To determine whether peri-ovulatory L-ornithine supplementation conveys similar reproductive benefits in old mice to those observed with peri-ovulatory putrescine supplementation (Tao et al., 2015).

2. Materials and Methods

2.1. Mice and Reagents

Animal protocols were approved by the Animal Care Committee of the Ottawa Hospital Research Institute and University of Ottawa (OHRI-1833 and OHRI-1834). All animals utilized in the study were purchased from Charles River (St-Constant, QC, Canada). Chemicals were from Sigma (St. Louis, MO, USA) unless otherwise indicated. L-ornithine monohydrochloride is of the BioReagent grade (Sigma, O-6503).

2.2. Superovulation Protocol

Female CF1 and C57BL/6 mice were treated utilizing a 5 IU/mouse intraperitoneal (IP) injection of equine chorionic gonadotropin (eCG, Sigma, G4527) to stimulate maturation of the ovarian follicles. Forty-six hours later, 5 IU/mouse of human chorionic gonadotropin (hCG, Sigma, C1063) was injected via IP injection to induce ovulation. After treatment with hCG, mice were given a dose of L-ornithine by oral gavage or sub-cutaneous injection (Figure 2). Dosages, routes of delivery, and delivery times are indicated in figure captions.

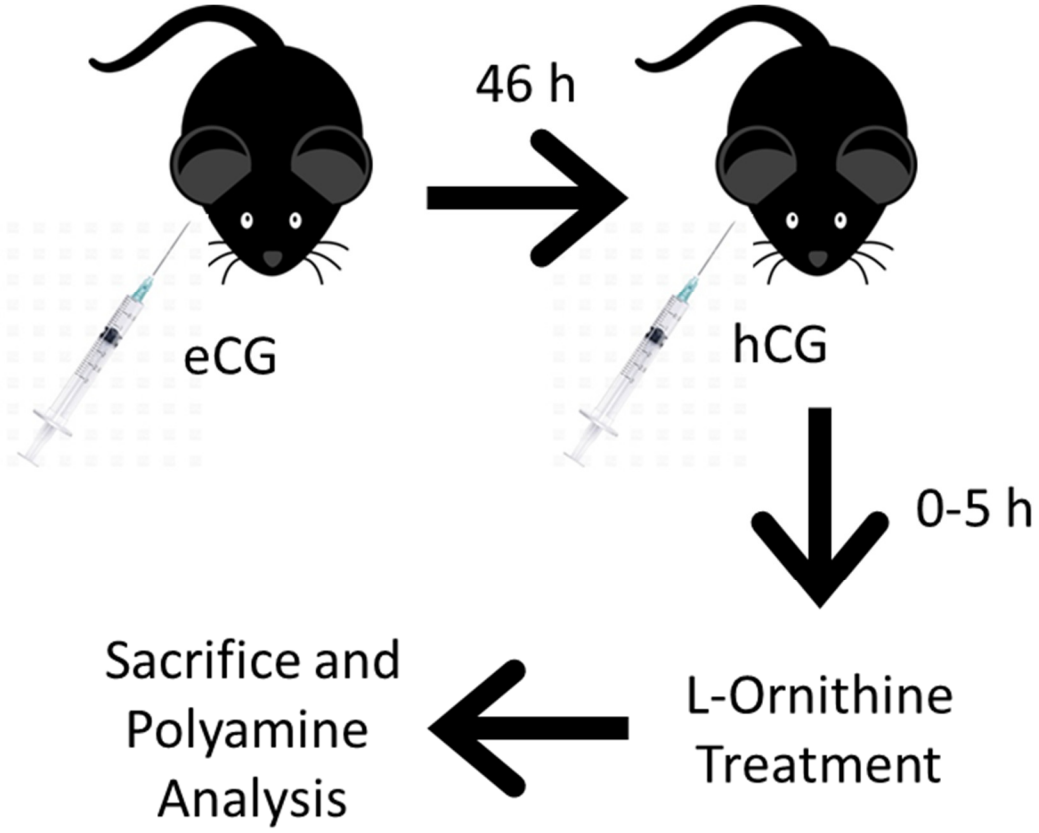

Figure 2: Diagrammatic representation of the superovulation and L-ornithine administration methodology utilized in the study.

2.3. Natural Estrus Cycle Tracking

Estrus state of young CF1 mice was analyzed by vaginal cell cytology according to Byers et al. (2012). Briefly, each morning at approximately 9:00 am, the vagina was gently flushed with 20 µL of phosphate buffered saline (PBS) using a 20 µL pipette. The cell suspension was then placed on a clean microscope slide and viewed under a microscope. Estrus stage was determined by the cell types present in vaginal smears.

Diestrus: Longest stage, lasting 48 to 72 hours (Cora et al., 2015). Cell suspension is composed of mostly leukocytes.

Proestrus: Stage lasts less than 24 hours (Cora et al., 2015). Cell suspension is composed of mostly nucleated epithelial cells, some cornified epithelial cells.

Estrus: Stage lasts between 12 and 48 hours (Cora et al., 2015). Cell suspension is composed of mostly cornified epithelial cells.

Metaestrus: Stage lasts less than 24 hours (Cora et al., 2015). Cell suspension is a mix of leukocytes, nucleated epithelial cells, and cornified epithelial cells.

Young CF1 mice determined to be in the proestrus phase were then given free access to 4% L-ornithine (w/v) in water at approximately 15:00. Mice were then sacrificed by cervical dislocation at approximately 02:00 the day following proestrus and their ovaries were removed. Ovarian polyamine concentrations were determined as stated below.

2.4. Polyamine Extraction

At the indicated times, mice were euthanized via cervical dislocation. Tissues of interest were then removed, placed in pre-weighed 1.5 mL micro-tubes, and flash frozen on dry ice. The tissues were then placed at -80°C until time of extraction.

All tissues were extracted utilizing homogenization in 5% trichloroacetic acid (TCA, Sigma, T-6399) in deionized water. Ovary samples were extracted with 20 µL of 5% TCA per mg of tissue. For the tissue distribution study (Table I), the ratios of 5% TCA to tissue weight were as follows: 10 µL per mg of tissue for uterus, spleen, kidney, liver and intestine; 5 µL per mg of tissue for stomach, lung, heart, brain, skin (ear), and muscle (leg).

Tissue samples were minced with surgical scissors in the indicated volumes of 5% TCA for one minute. The minced tissue was then homogenized three times by sonication with a sonic dismembrator (Fisher Sonic Dismembrator Model 300) at 35% of maximum power for one minute. Tissue homogenates were then centrifuged in a table top micro-centrifuge at 10,000 x g for 10 minutes at 4°C (Microfuge 22R Centrifuge, Beckman Coulter).

The supernatants from tissue homogenates were transferred with a micropipette to a new 1.5 mL micro-tube. Using a disposable pipette, diethyl ether (Sigma, 296082) was added to the supernatant to increase the total volume to approximately 1.5 mL. Samples were vortexed (Vortex Genie 2, VWR) at maximum power for one minute and centrifuged at 10,000 x g for one minute at 4°C. The lower aqueous phase was then removed by micropipette and transferred to a new 1.5 mL micro-tube and the diethyl ether addition was repeated. 100 µL of extract was then diluted with 900 µL of water for analysis via ion chromatography.

2.5. Ion Chromatography and Determination of Tissue Polyamine Concentrations

The diluted polyamine extract was directly subjected to ion chromatography on a Dionex Ion Chromatography system equipped with an Ion Pac CS18 cation exchange column

(http://www.dionex.com/en-us/products/columns/ic-rfic/cation-packed/ionpac-cs18/lp-73230.html). Samples were eluted from the column by increasing concentrations (3-45 mM) of methanesulfonic acid (MSA, Sigma, 64280). The eluate was subjected to an online ERS^{TM}500 suppressor for cations, in order to eliminate all anions including MSA, before a conductivity detector visualizing all cations. Levels of polyamines were measured by the conductivity of peak areas in μSeimens/s calibrated against a standard curve derived from a dilution series of four polyamines (putrescine, histamine, spermidine, and spermine).

2.6. Breeding and Analysis of Births

Old C57BL/6 mice (9.5-13 months of age) were randomized in Microsoft Excel and assigned to control (water) or treatment with 1% L-ornithine monohydrochloride w/v, simply 1% ornithine, 4% ornithine, or 1% putrescine groups. Female mice were given free access to control or drug-containing water, as well as male bedding two days prior to mating. Exposure to male bedding synchronises the estrus cycles and is known as the Whitten Effect (Whitten, 1958). Young BDF-1 males were then co-housed with females for four consecutive days. Water consumption was tracked daily for all test groups and water was refreshed every two days. After the four days of mating, female mice were given regular water and housed individually. Mice were then checked daily for births from 19 days post coitum (dpc) to 25 dpc when the four possible conception dates were taken into consideration. The number of pups birthed was recorded.

2.7. Implantation and Resorption Experiment

Old C57BL/6 mice (10.5 months of age) were given male bedding and free access to control or 4% L-ornithine hydrochloride (w/v) in water two days prior to mating. Young male BDF-1 mice were then reintroduced to the females, control or 4% ornithine water was

refreshed, and mice were mated until a copulation plug was observed or for four consecutive days. Each morning mice were examined for presence of copulation plug, if a copulation plug was observed we considered this time 0.5 dpc. Mice with copulation plugs were removed from the breeding cage and given normal water. At 9.5 dpc, mice were sacrificed via cervical dislocation and the uterus was removed. Implantation sites were counted, and placentas were dissected according to Pereira et al. (2011). Individual implantation sites were classified as:

Normal Fetuses: fetuses that display typical morphology at 9.5 dpc corresponding to Theiler stage 15 (Theiler, 1989).

Resorptions: Implantation sites that contain no intact fetus after placental dissection.

2.8. Statistical Analysis

Data are provided at means ± SEM. Data from polyamine analyses were analysed via Student's t-test comparing treatment groups to controls, one-way ANOVA with Dunnett's multiple comparison test comparing treatment to controls, or one-way ANOVA with Tukey's post-hoc test. Data from mating analyses of pups birthed were analysed via chi-square goodness of fit test and Poisson linear regression. Litter size and implantation data were analysed by Student's t-test. Resorption data were analysed by Fisher's exact test (two tailed). Significance was set at $p < 0.05$ and data were analysed using GraphPad Prism v. 7.00 (GraphPad Software Inc.).

3. Results

3.1. L-Ornithine Increases Ovarian Putrescine Concentrations

We employed ion chromatography and direct conductivity (ions) detection to determine what changes, if any, occur in ovarian polyamine concentrations in young and old mice following supplementation with L-ornithine. The transient rise of ODC during oocyte maturation is triggered by a surge of LH, or hCG injection in the lab (Maudsley and Kobayashi, 1974) and ODC activity produces peak putrescine concentrations approximately 5h after hCG injection (Maudsley and Kobayashi, 1974; Tao et al., 2015). We thus wished to determine if exogenous L-ornithine was capable of eliciting increases in ovarian putrescine concentrations after hCG administration. Super-ovulated young CF1 mice were given a 500 mg/kg dose of L-ornithine or phosphate buffered saline (PBS, vehicle) as control. This dose was 5% of the LD_{50} for L-ornithine (Breglia et al., 1973) and was therefore deemed safe for the animal. L-ornithine significantly increased ovarian putrescine concentrations resulting in approximately three-fold higher ovarian putrescine concentrations compared to control mice. In contrast, L-ornithine supplementation did not significantly alter spermidine and spermine concentrations (figure 3A).

Similar L-ornithine supplementation was also given to mice primed with only eCG. In this case, L-ornithine administration did not elicit any large increase in ovarian putrescine concentrations regardless of administration time (1-5 h) prior to sacrifice (figure 3B). Concentrations of spermidine and spermine did not change significantly either following L-ornithine administration. These results suggest that L-ornithine can only increase ovarian putrescine concentrations in the presence of ODC activity (i.e. after hCG injection).

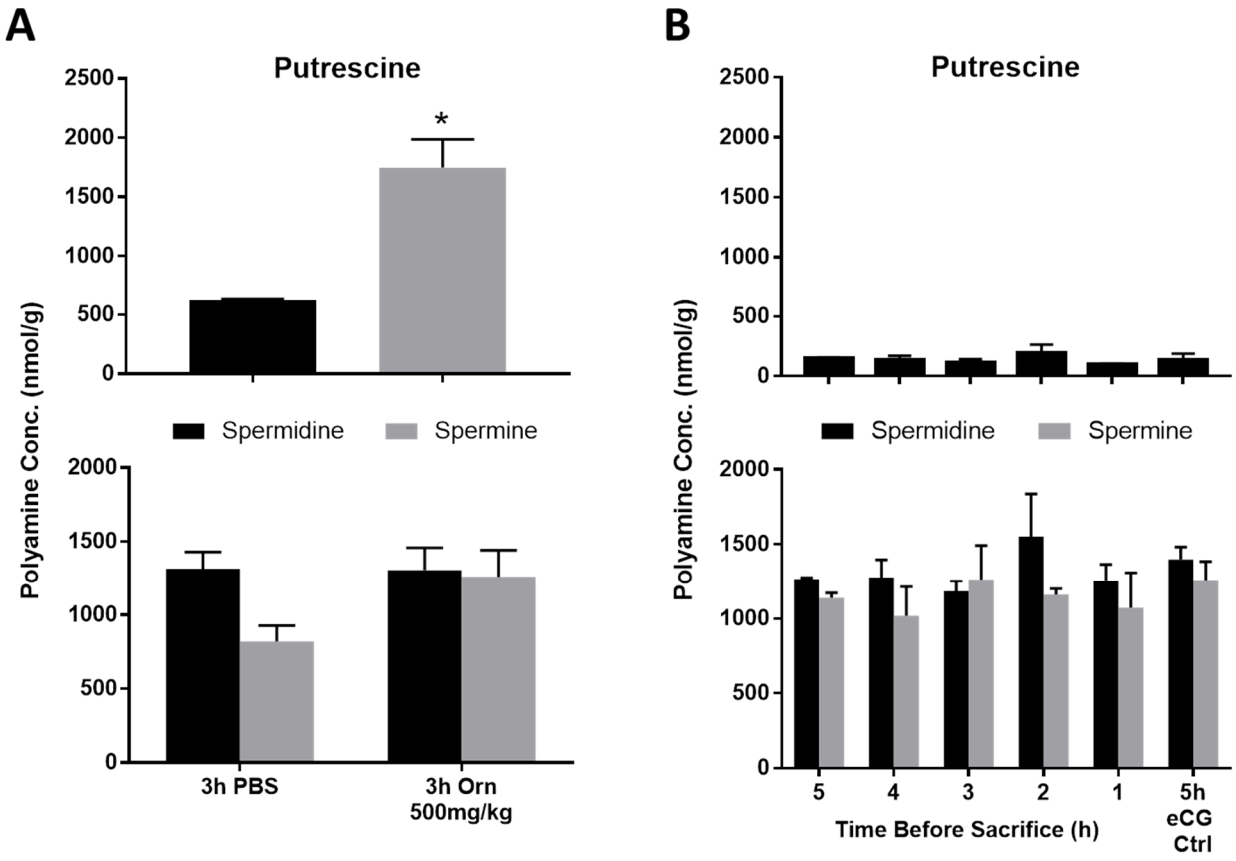

Figure 3: L-ornithine increases ovarian putrescine in young mice only after both eCG and hCG treatment. (A) Young CF1 mice (n=3 per group) were primed treated with eCG and hCG. Mice were then treated with PBS or a 500 mg/kg dose of L-ornithine via oral gavage 3h after hCG administration. Ovarian polyamine concentrations were determined 5h after hCG treatment. Data are presented as means ± SEM. Asterisks indicate $p < 0.05$ versus control determined by Student's t-test. (B) Young CF1 mice (n= 2 per group) primed with eCG only. Following eCG priming, mice received a 500 mg/kg dose of L-ornithine via oral gavage 1-5h prior to sacrifice. Mice on the far right received PBS via oral gavage. Ovarian polyamine concentrations were determined. Data are presented as means ± SEM.

We next desired to know if exogenous L-ornithine could increase peri-ovulatory ovarian putrescine concentrations in aged mice. Older C57BL/6 mice were primed with eCG followed by hCG. As shown previously by Tao et al. (2015), hCG treatment increased putrescine concentrations in the ovaries during ovulation (figure 4, CTRL). L-ornithine supplementation together with hCG injection increased ovarian putrescine concentrations approximately three-fold (figure 4, ORN) over those induced by hCG treatment alone. However, this difference was only significant ($p < 0.05$) at three and five hours after hCG administration. Ovarian spermidine and spermine concentrations did not differ significantly between control and L-ornithine treated animals (figure 4). These results support our hypothesis that peri-ovulatory supplementation of L-ornithine can increase ovarian putrescine in aged mice.

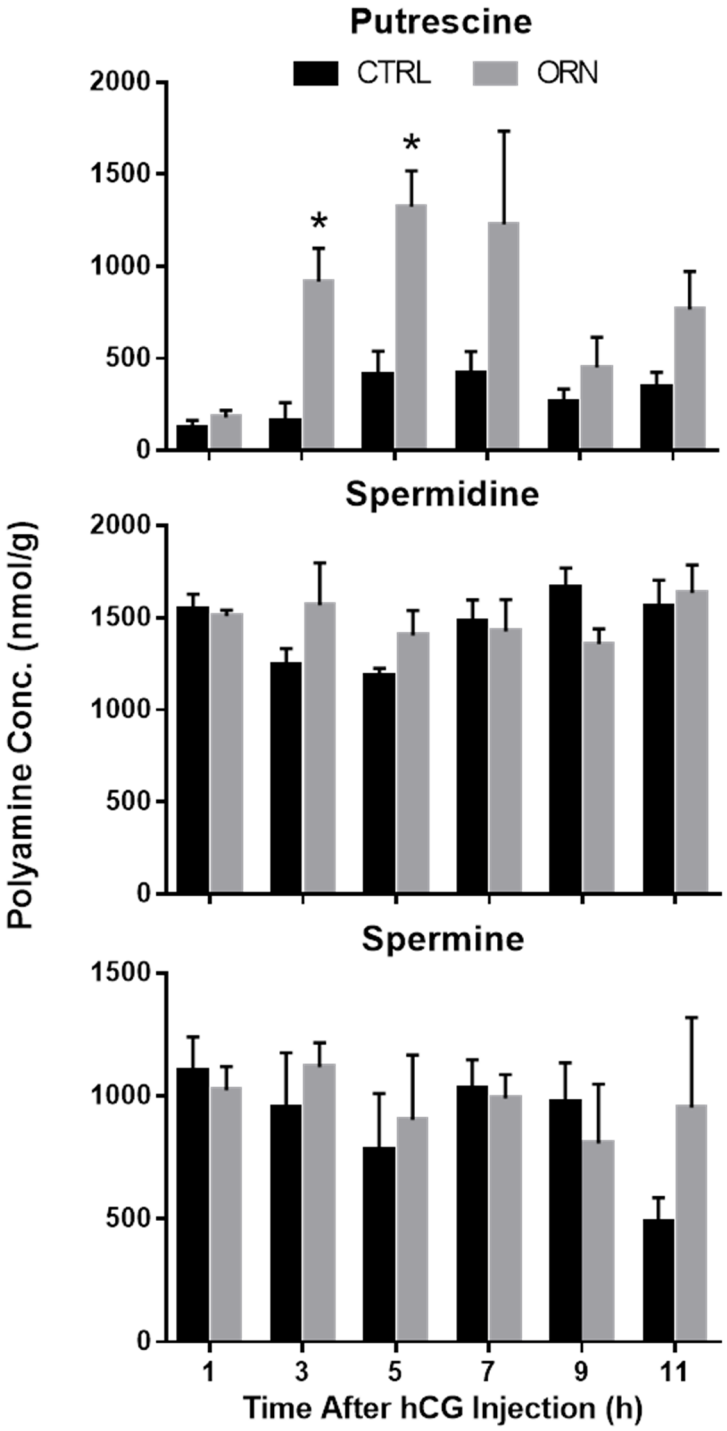

Figure 4: L-ornithine increases ovarian putrescine in old mice treated with eCG and hCG. 10-month-old C57BL/6 mice (n=3 per group) were treated with eCG and hCG. Mice were sacrificed at the indicated time after hCG injection. One hour prior to sacrifice, the mice were given PBS (CTRL) or L-ornithine in PBS (500 mg/kg) by oral gavage. Ovarian polyamines were then analysed and quantified. Data are presented as means ± SEM. Asterisks indicate $p < 0.05$ versus control determined by Student's t-test.

3.2. Determining the Minimum Dose of L-Ornithine Required for Restoring Peri-Ovulatory Ovarian Putrescine Levels in Older Mice

L-ornithine supplementation, at 500 mg/kg, raised peri-ovulatory ovarian putrescine in older mice to levels greater than those found in young control animals (figures 3 and 4). Our goal was to restore ovarian peri-ovulatory putrescine concentrations in older mice roughly to the levels observed in young mice. Thus, older mice were primed with eCG followed by hCG then treated with varying doses of L-ornithine to examine the effect of different concentrations of L-ornithine on ovarian putrescine concentrations. L-ornithine supplementation increased ovarian putrescine concentrations, in a dose-dependent manner, with 250 and 500 mg/kg doses reaching statistical significance (figure 5). In contrast, spermidine concentrations remained relatively unchanged regardless of the dose of L-ornithine administered. Spermine concentrations were less consistent, but only the highest dose of L-ornithine resulted in significantly different spermine concentrations from controls. These results further strengthen our hypothesis that observed increases in ovarian putrescine concentrations are the result of peri-ovulatory L-ornithine supplementation.

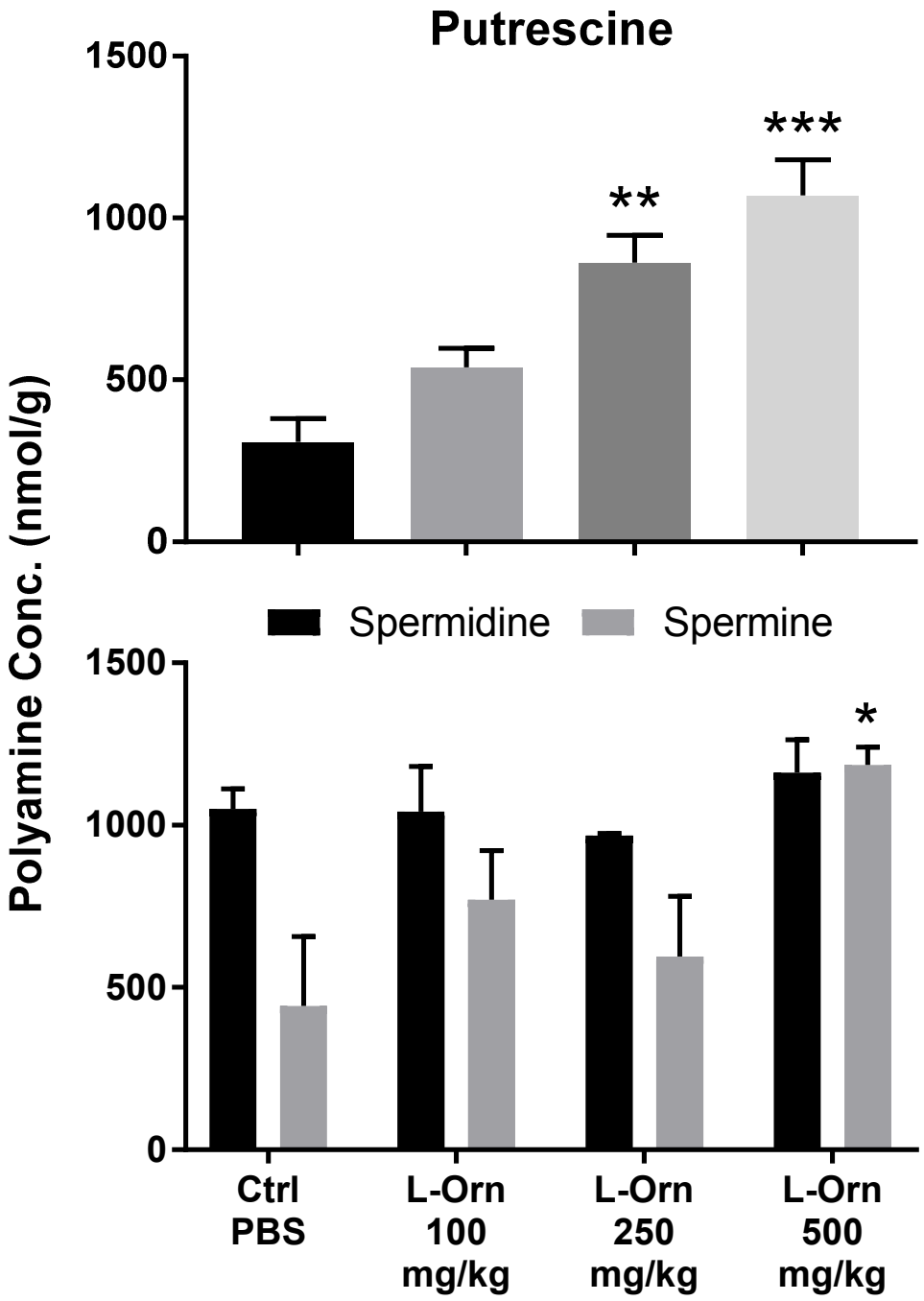

Figure 5: L-ornithine dose-dependently increases ovarian putrescine concentrations in older mice. 10-month-old C57BL/6 mice (n=3 per group) were treated with both eCG and hCG. Mice were then sub-cutaneously injected with PBS (control) or 100, 250, or 500 mg/kg doses of L-ornithine 4h after hCG treatment. Ovarian polyamines were analysed and quantified 1h later. Data are presented as means ± SEM. Asterisks represent statistical significance, as determined by a one-way ANOVA with Dunnett's multiple comparison test versus control values as follows: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

3.3. L-Ornithine Specifically Increases Ovarian Putrescine Concentrations

Given the systemic nature of our treatments, we sought to examine the tissue distribution of putrescine following peri-ovulatory L-ornithine or putrescine supplementation. Older C57BL/6 mice primed with eCG and hCG. We then treated the mice with PBS as a control, 100 mg/kg of putrescine, or 500 mg/kg of L-ornithine. The polyamine content of various tissues was determined 1h later. All three polyamines were detected in the tissue samples with the exception of putrescine not being detectable in the muscle of control mice (table I). Compared to PBS-treated mice, putrescine treatment increased putrescine concentrations in most tissues, often dramatically. For example, putrescine treatment increased ovarian putrescine approximately two-fold, but increased kidney and liver putrescine concentrations by approximately 20- and 50-fold, respectively, compared to PBS-treated controls. In contrast, L-ornithine treatment only increased ovarian putrescine concentrations significantly by approximately four-fold compared to PBS-treated control mice. All other tissues in L-ornithine treated animals had putrescine concentrations that were not significantly different from PBS-treated control animals.

Spermidine and spermine concentrations following putrescine or L-ornithine treatment did not vary significantly compared to PBS-treated controls in most tissues examined. There were some exceptions. However, when compared to the drastic changes observed in tissue putrescine concentrations, spermidine and spermine concentrations varied between putrescine or L-ornithine treated animals and PBS-treated animals by approximately 15-20% (table I). These results support our hypothesis that peri-ovulatory L-ornithine supplementation specifically increases ovarian putrescine due to the specific increase of ODC activity in the ovaries during ovulation (Kaye et al., 1973; Kobayashi et al., 1971). By

contrast, putrescine supplementation increased putrescine levels in all tissues non-discriminately.

Table I: Polyamine tissue distribution in old mice following peri-ovulatory putrescine or L-ornithine supplementation.

Polyamine (nmol/g)	Tissue	Group		
		Control	Putrescine	Ornithine
Putrescine	Ovaries	308.18 ± 71.99	695.87 ± 94.84*	1069.52 ± 109.90**
	Uterus	31.00 ± 9.76	234.69 ± 45.33**	62.25 ± 27.79
	Spleen	541.15 ± 274.70[1]	448.66 ± 36.03	245.45 ± 44.44
	Kidney	33.59 ± 12.23	642.40 ± 151.10**	56.21 ± 20.65
	Liver	12.54 ± 12.54	754.64 ± 143.90**	41.22 ± 21.00
	Intestine	150.53 ± 31.43	517.97 ± 73.32**	137.61 ± 40.64
	Stomach	19.43 ± 6.65	125.78 ± 16.09**	33.24 ± 13.87
	Lung	32.47 ± 2.88	359.96 ± 28.69****	45.77 ± 13.82
	Heart	4.83 ± 4.83	161.75 ± 25.08***	16.44 ± 10.12
	Brain	3.10 ± 3.10	17.20 ± 4.02	47.93 ± 41.06[1]
	Skin (Ear)	74.62 ± 11.18	262.45 ± 65.47*	61.17 ± 17.06
	Muscle (Leg)	n.d.	14.35 ± 14.35	9.44 ± 9.44
Spermidine	Ovaries	1050.53 ± 61.95	1022.62 ± 108.00	1161.42 ± 101.10
	Uterus	1052.00 ± 40.54	1092.27 ± 102.70	1210.78 ± 24.48
	Spleen	1322.59 ± 118.00	1214.65 ± 25.93	1153.12 ± 60.86
	Kidney	395.08 ± 14.91	420.51 ± 29.84	429.52 ± 16.22
	Liver	903.77 ± 49.74	898.31 ± 114.80	1004.67 ± 27.67
	Intestine	1531.97 ± 59.92	1482.49 ± 112.70	1381.1 ± 37.78
	Stomach	519.74 ± 3.72	627.02 ± 22.70	528.73 ± 71.80
	Lung	476.77 ± 8.53	529.85 ± 33.97	460.22 ± 27.09
	Heart	161.53 ± 6.31	183.97 ± 11.98	183.34 ± 19.56
	Brain	329.65 ± 35.01	466.68 ± 70.47	446.47 ± 107.20
	Skin (Ear)	607.25 ± 41.05	600.89 ± 62.87	433.50 ± 75.31
	Muscle (Leg)	75.09 ± 10.44	100.76 ± 17.54	97.93 ± 5.58
Spermine	Ovaries	442.63 ± 214.40	563.92 ± 172.70	1185.90 ± 54.54*
	Uterus	524.05 ± 91.28	403.70 ± 44.09	697.03 ± 19.78
	Spleen	1123.13 ± 102.30	897.13 ± 42.68	1026.19 ± 37.83
	Kidney	1103.40 ± 45.79	1010.72 ± 64.44	1056.01 ± 67.61
	Liver	989.62 ± 83.93	983.65 ± 123.50	943.19 ± 5.53
	Intestine	1037.42 ± 106.50	1009.82 ± 70.72	962.02 ± 17.75
	Stomach	494.24 ± 39.08	533.98 ± 8.99	489.10 ± 91.45
	Lung	507.75 ± 13.30	481.22 ± 69.75	416.88 ± 22.14
	Heart	355.23 ± 19.04	451.71 ± 15.50*	384.38 ± 26.38
	Brain	338.02 ± 43.59	374.68 ± 39.77	269.15 ± 26.67
	Skin (Ear)	338.91 ± 60.21	363.26 ± 83.70	299.05 ± 31.04
	Muscle (Leg)	126.56 ± 36.85	172.52 ± 16.75	116.36 ± 19.82

10-month-old C57BL/6 mice (n=3 per group) were primed with eCG followed by hCG. 4h after hCG treatment, mice were sub-cutaneously injected with PBS (control), 100 mg/kg

putrescine, or 500 mg/kg L-ornithine. Tissue polyamine concentrations were analysed and quantified 1h later. Polyamine concentrations are shown as means ± SEM. Statistical significance as determined by a one-way ANOVA with Dunnett's multiple comparison test versus control values are represented as follows: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. n.d., not detected. [1] Inflated means caused by abnormally high values from a single mouse.

3.4. Exogenous L-Ornithine has a Limited Effective Period

As L-ornithine specifically and dose-dependently increased ovarian putrescine, we wished to determine the period of a single dose of L-ornithine could increase ovarian putrescine concentrations. To explore this, older C57BL/6 mice were primed with eCG and hCG. The mice then received a dose of L-ornithine one to five hours prior to sacrifice and polyamine determination (figure 6). L-ornithine appeared to increase ovarian putrescine at all time points except when administered five hours before sacrifice. When the data were analyzed by one-way ANOVA, it was evident that L-ornithine administered one hour before sacrifice yielded the most and significant increase in ovarian putrescine of approximately three-fold compared to PBS-treated controls. L-ornithine administered between two and four hours before sacrifice yielded increases of ovarian putrescine of approximately 1.5- to 2-fold compared to PBS-treated controls, but the increase did not reach statistical significance. It should be noted that ovarian putrescine levels two to four hours after a bolus of L-ornithine administration were also not significantly different from that one hour after L-ornithine administration (figure 6). Therefore, exogenous L-ornithine appeared to sustain ovarian putrescine concentrations, at least partially, for several hours in mice. These results are consistent with published data suggesting that exogenous L-ornithine can remain in circulation for several hours in humans (Kato et al., 1987).

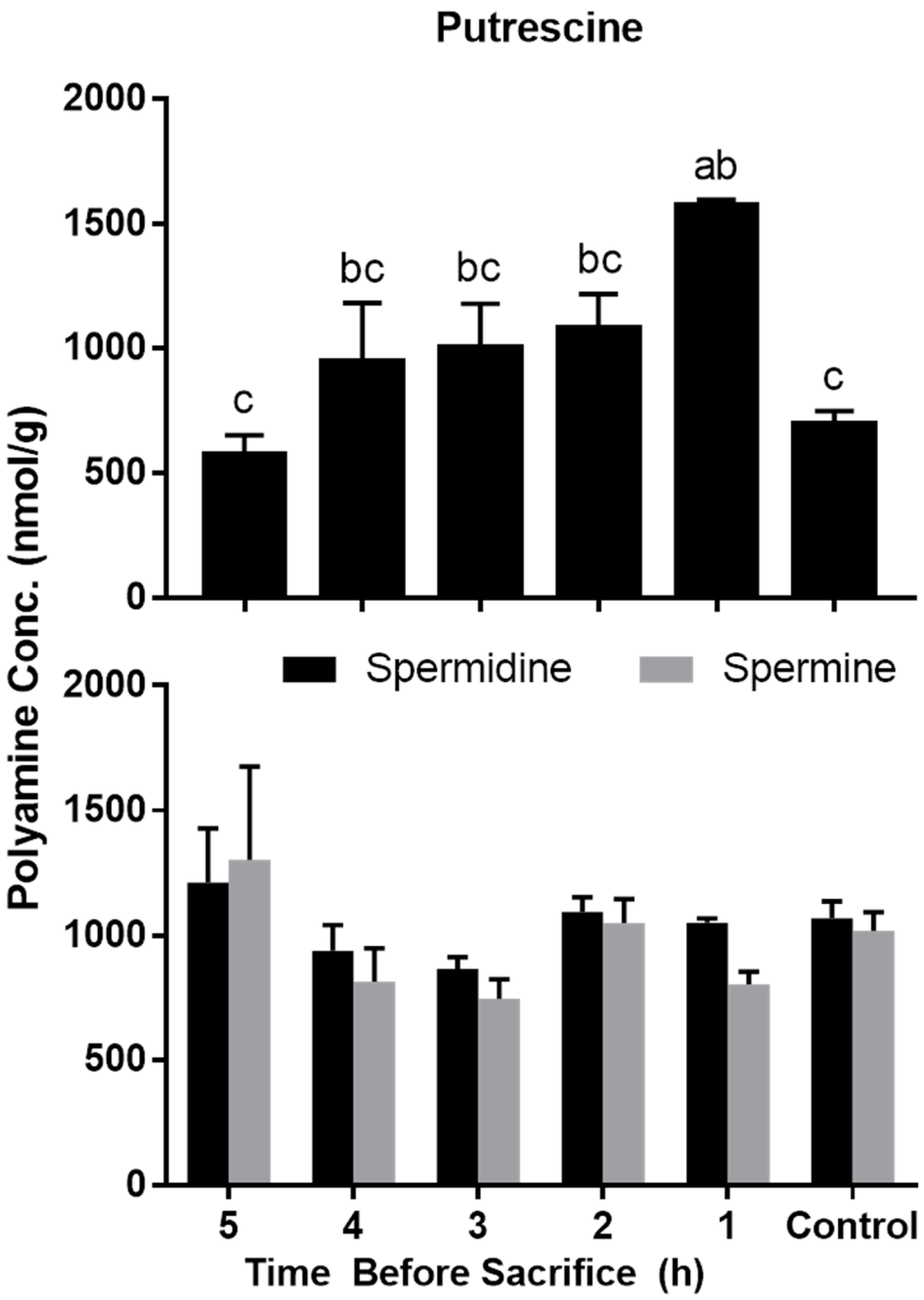

Figure 6: L-ornithine supplementation at various times following hCG injection. 10-month old C57BL/6 mice (n=3 per group) were treated with eCG and hCG. At the indicated time before sacrifice, mice were given a 500 mg/kg dose of L-ornithine via oral gavage. Control mice (right) received no treatment. Ovarian polyamine concentrations were analysed and quantified 5h after hCG treatment. Data are presented as means ± SEM. Statistics were determined via one-way ANOVA and Tukey's post-hoc test. Bars without a common letter are significantly different, $p < 0.05$.

3.5. 1% L-Ornithine Supplementation During Mating

To investigate the effect of L-ornithine on reproductive success, we started with 1% L-ornithine supplemented in drinking water (w/v). We chose 1% L-ornithine supplementation as reproductive benefits have been observed from supplementation of 1% putrescine (w/v) in drinking water (Tao et al., 2015). We thus wished to see if 1% L-ornithine in drinking water provided similar reproductive benefits to aged mice.

Female C57BL/6 retired breeders (10-months of age) were bred with young male BDF-1 mice. Male and female mice were randomized for each round of breeding and assigned to two groups: control or 1% L-ornithine (w/v) in drinking water. Female mice were then given male bedding and free access to water or L-ornithine containing drinking water for two days. Afterward, the males were introduced to the cage and co-housed with the females for four consecutive days. The males were removed, and the females were single housed and were given regular drinking water. The females were examined for births from 19 dpc to 25 dpc considering the four possible conception dates. The number of pups birthed was recorded and the results are presented in table II. Detailed tracking of individual mice across all 1% L-ornithine mating can be found in the appendix (table V).

Mice treated with 1% L-ornithine in drinking water consumed approximately 10% more water compared to control mice (figure 7A). Supplementation of 1% L-ornithine in drinking water did not significantly alter the number of litters or the litter size compared to control mice at any of the time points analysed. Given equal numbers of mice in each of the two groups, equal numbers of pups would be expected from each group if 1% L-ornithine supplementation in drinking water has no effect. Our data appeared to show that as the animals aged, more pups were produced in L-ornithine groups compared to aged matched

controls (11-13 months). We thus preformed chi-square goodness of fit analyses (McHugh, 2013; Rana and Singhal, 2015) on the number of pups produced in the control and L-ornithine treated mice for each of the four age groups. The rationale of the Chi-square goodness of fit test is to determine if the frequencies observed vary from the expectations of the null hypothesis (i.e. equal numbers of pups in control and L-ornithine treated mice). Chi-square goodness of fit analysis indicated that 1% L-ornithine significantly increased the number of pups at 11 and 13 months of age by approximately 1.5- and 2.5-fold respectively, but not 9.5 or 12 months of age (table II). When the combined total pups from 9.5 to 13 months were compared between the two groups, no significant difference was found. Furthermore, analysis of the data across time by Poisson linear regression found significantly fewer pups born with time in both groups, but no significant effect of 1% L-ornithine treatment was detected compared to control at any age. These results reveal that compared to 1% putrescine supplementation in drinking water, 1% L-ornithine supplementation in drinking water has a small effect on reproductive outcome at best. These results seem to indicate that 1% L-ornithine supplementation in drinking water may be insufficient to provide reproductive benefit to mice.

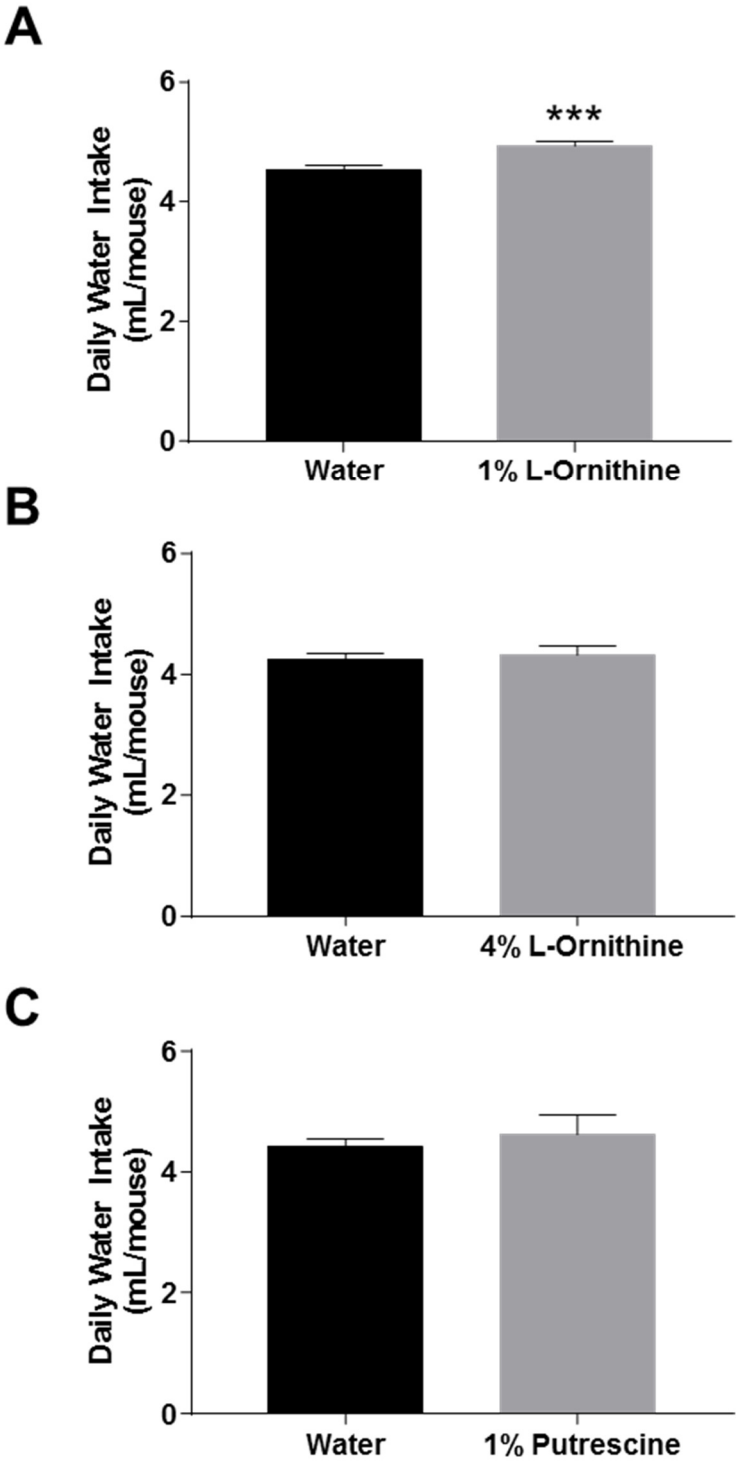

Figure 7: Average daily water intake of aged mice supplemented during mating with (A) water (n=336) and 1% L-ornithine (n=340), (B) water (n=75) and 4% L-ornithine (n=70), and (C) water (n=60) and 1% putrescine (n=58). Data are presented as means ± SEM. Statistical significance as determined by Student's t-test is represented as follows: *** p <0.001.

Table II: 1% L-ornithine treatment in chronologically bred mice has limited reproductive benefit.

Age	Treatment	N	Litters (% mice)	Litter Size ± SEM	Total Pups	p (Total Pups)
9.5 Months	Control	68	34 (50.0)	4.3 ± 0.5	145	0.1593
	1% Ornithine	68	29 (42.6)	4.2 ± 0.5	122	
11 Months	Control	67	20 (29.9)	3.5 ± 0.5	70	0.0257
	1% Ornithine	66	22 (33.3)	4.5 ± 0.5	99	
12 Months	Control	46	5 (10.9)	3.6 ± 1.1	18	0.3545
	1% Ornithine	45	8 (17.8)	3.0 ± 0.5	24	
13 Months	Control	43	5 (11.6)	1.4 ± 0.2	7	0.0412
	1% Ornithine	44	6 (13.6)	2.8 ± 0.9	17	
All Ages Combined	Control	224	64 (28.6)	3.8 ± 0.3	240	0.3261
	1% Ornithine	223	65 (29.1)	4.0 ± 0.3	262	

Control and peri-ovulatory L-ornithine treated (1% w/v in drinking water) C57BL/6 mice were chronologically mated with young BDF-1 males and examined for birthing, recorded as litter size and total number of pups. Only mice giving birth to at least one pup were included in the litter size calculations. Statistics were determined using the chi-square goodness of fit test; significance was set at $p < 0.05$.

3.6. 4% L-Ornithine Supplementation During Mating

As 1% L-ornithine supplementation did not provide the reproductive benefit we expected, we further increased the concentration of L-ornithine from 1% to 4% in drinking water. In practice, 4% L-ornithine (w/w) has been previously supplemented to rats in food with no adverse effects observed (Sakamoto et al., 2015). Since mice consume roughly equal amounts of food and water (Bachmanov et al., 2002), they should consume roughly the same amount of L-ornithine in drinking water or in food.

Therefore, we bred our aged C57BL/6 mice in a similar fashion to the previous experiments examining the effect of 1% L-ornithine on reproductive outcome. The mice were randomized between control and 4% L-ornithine in drinking water and mated for four consecutive days with young BDF-1 males. We then checked the females for births from 19 dpc to 25 dpc considering the four possible conception dates. The number of pups birthed was recorded and the results are presented in table III. A detailed breakdown of the reproductive history of our mice across 4% L-ornithine breeding is available in the appendix (table VI).

The presence of L-ornithine in drinking water was not observed to affect water consumption (figure 7B). However, 4% L-ornithine supplementation appeared to adversely affect fertility of these older mice. At 10-months of age, a slightly reduced number of litters were found in the L-ornithine treated group compared to the control group (8 vs. 11, or 38% vs. 52%, respectively) when the litter size was similar. At 11.5-months of age, significantly smaller litter size was observed in the L-ornithine group compared to the control group when the number of litters was identical. More revealing perhaps was the smaller number of total pups observed in L-ornithine treated mice at both ages compared to control. Chi-square

goodness of fit analysis revealed a significant decrease in the number of pups birthed at 11.5 months of age compared to age-matched controls (table III). A comparison of the combined total pups also revealed a significant reduction in the 4% L-ornithine supplemented group.

L-ornithine administered orally specifically increased putrescine concentrations in the ovaries in mice injected with hCG (figure 10) and yet peri-ovulatory L-ornithine supplementation had either no effect (1%) or appeared to negatively affect the fertility of these retired breeders. To determine if these C57BL/6 retired breeders, which were purchased from Charles River, could indeed benefit from peri-ovulatory putrescine supplementation as their counterparts purchased from Taconic (Tao et al., 2015), we performed 1% putrescine supplementation and mating experiments on the same mice. We randomized the mice once again between control and 1% putrescine in drinking water. 1% putrescine in drinking water was not observed to affect water consumption (figure 7C). Putrescine treatment was observed to more than double the number of pups and increased the litter size by approximately 1.5-fold compared to control mice (table III). The results we observed are similar to those presented in Tao et al. (2015) showing an approximate doubling of the number of pups in putrescine-treated mice. Our results indicate that L-ornithine supplementation, at least when administered in drinking water (1% or 4%) during ovulation and mating, did not provide any reproductive benefit to aged mice. These results also serve to confirm the reproductive benefit of putrescine supplementation in aged mice (Tao et al., 2015; Tao and Liu, 2013).

Table III: 4% L-ornithine treatment has undesirable effects on reproductive potential in aged mice.

Age	Treatment	N	Litters (% Mice)	Litter Size ± SEM	Total Pups	p (Total Pups)
10 Months	Control	21	11 (52.4)	4.2 ± 0.6	46	0.0874
	4% Ornithine	21	8 (38.1)	3.9 ± 0.8	31	
11.5 Months	Control	31	7 (22.6)	5.0 ± 0.9	35	0.0195
	4% Ornithine	31	7 (22.6)	2.6 ± 0.3*	18	
10 and 11.5 Months Combined	Control	52	18 (34.6)	4.5 ± 0.5	81	0.005
	4% Ornithine	52	15 (28.8)	3.3 ± 0.5	49	
12.5 Months	Control	31	4 (12.9)	3.3 ± 0.6	13	0.0022
	1% Putrescine	31	7 (22.6)	4.9 ± 0.9	34	

Control and peri-ovulatory L-ornithine treated (4% w/v in water) and putrescine (1% w/v in water) C57BL/6 mice were chronologically mated with young BDF-1 males and examined for birthing, recorded as litter size and total number of pups. Only mice giving birth to at least one pup were included in the litter size calculations. Statistics were determined using the chi-square goodness of fit test on total pups; significance was set at $p < 0.05$. Asterisks indicate statistical significance versus control determined by Student's t-test, $p < 0.05$.

3.7. Effect of 4% L-Ornithine Supplementation on Implantation and Resorption

We wished to investigate the apparent adverse effect of 4% L-ornithine supplementation more closely. To do this, aged mice were bred in a similar fashion to previous experiments; however, they were checked daily for the presence of a copulation plug. Plugged female mice were removed from males, given regular drinking water, and considered 0.5 dpc. Plugged mice were then sacrificed at 9.5 dpc and pregnancy status was determined. If the mice were pregnant, the uterus was removed, and the implantation sites were counted and dissected (figure 8A). Fetuses were classified as normal if they displayed typical morphology at 9.5 dpc corresponding to Theiler stage 15 (Theiler, 1989) or resorbed if there was no intact fetus in the placenta (figure 8B).

Supplementation of 4% L-ornithine in drinking water did not affect the number of mice that became plugged or pregnant indicating that L-ornithine does not disrupt mating behavior or pregnancy rates. However, mice supplemented with 4% L-ornithine had 30% fewer implantation sites compared to control mice ($p < 0.05$). Among the implants found on 9.5 dpc, the proportion of normally developing or resorbed implantation sites was similar in the two groups (table IV). A detailed breakdown of the number of normally developing and resorbed fetuses can be found in the appendix (table VII). A reduction of implantation sites could be due to a reduction in the number of ovulated eggs, reduced fertilization rates, or reduced implantation rates.

Table IV: 4% L-ornithine supplementation decreases the number of implantations in aged mice.

Group	N	Plugs (% Mice)	Pregnancies (% Mice)	Total Implants	Average Implants ± SEM	Normal Implants (%)	Resorbed Implants (%)
Control	30	16 (53.3)	9 (30.0)	82	9.1 ± 0.6	63 (76.8)	19 (23.2)
4% L-Ornithine	30	19 (63.3)	11 (36.7)	59	5.4 ± 1.1*	48 (81.4)	11 (18.6)

10-month-old control and peri-ovulatory L-ornithine treated (4% w/v) C57BL/6 mice were mated with young BDF-1 males and examined for copulation plugs for 4 days. Mice were sacrificed at 9.5 dpc for pregnancy. Implantation sites were counted and dissected to examine fetal status. Asterisks indicate statistical significance versus control determined by Student's t-test, $p < 0.05$.

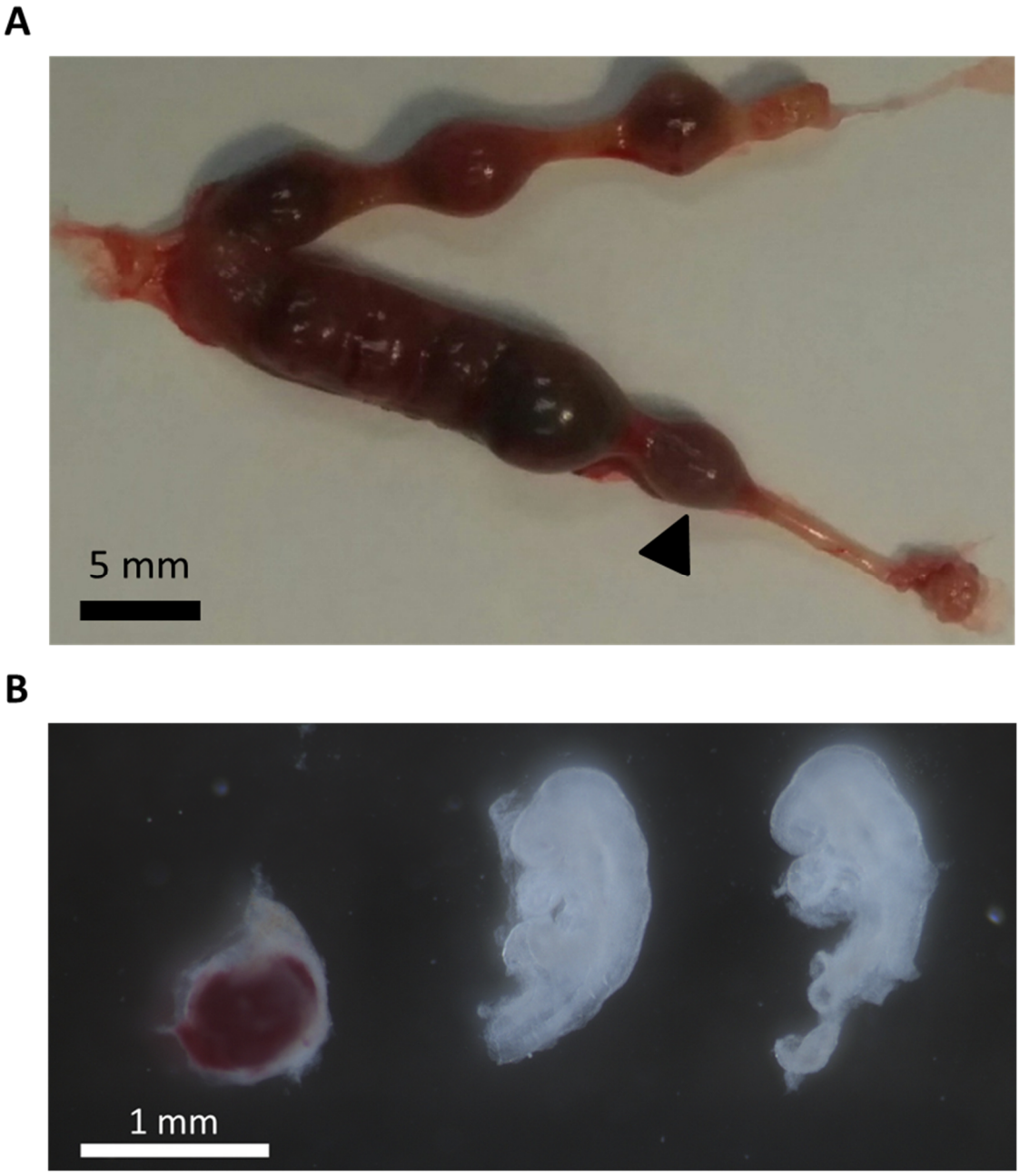

Figure 8: Determination of pregnancy, number of implantations, and fetal status in 10.5-month-old C57BL/6 mice treated with (n=30) or without (n=30) 4% L-ornithine. (A) Pregnancy status and number of implantation sites was determined by uterine dissection. A uterus from a 4% L-ornithine treated mouse contains 8 normally developing implants and 1 resorption site (black triangle). (B) Placental dissection was utilized to determine fetal developmental state. Two normally developing fetuses and one resorbed littermate (left) from a 4% L-ornithine treated mouse.

3.8. Analysing Ovarian Polyamines Following L-Ornithine Supplementation in Drinking Water

We investigated the efficacy of L-ornithine supplemented in water to increase ovarian putrescine concentrations during the natural estrus cycle. Peak putrescine concentrations have been observed to occur at approximately 02:00 on the day of estrus in mice (Liu et al., 2016a) and rats (Fozard et al., 1980c). We assessed the estrus status of young CF1 mice utilizing vaginal cell cytology (figure 9). Mice found to be in the proestrus phase (predominantly nucleated epithelial cells in the vaginal lavage) of the estrous cycle were then divided between control and 4% L-ornithine (w/v) drinking water groups in the late afternoon. Mice were then sacrificed at 02:00 the following morning (the morning of estrus). Ovaries were excised and subjected to polyamine analyses. We found slightly higher putrescine concentrations in the ovaries of mice in the L-ornithine group compared to control mice, but the difference was not significant (figure 10). Concentrations of spermidine and spermine were very similar in the two groups. Given the much greater variations in estrus cycle regularity (Nelson et al., 1982), and the prohibitive cost of older mice, we did not attempt similar analyses utilizing retired breeders.

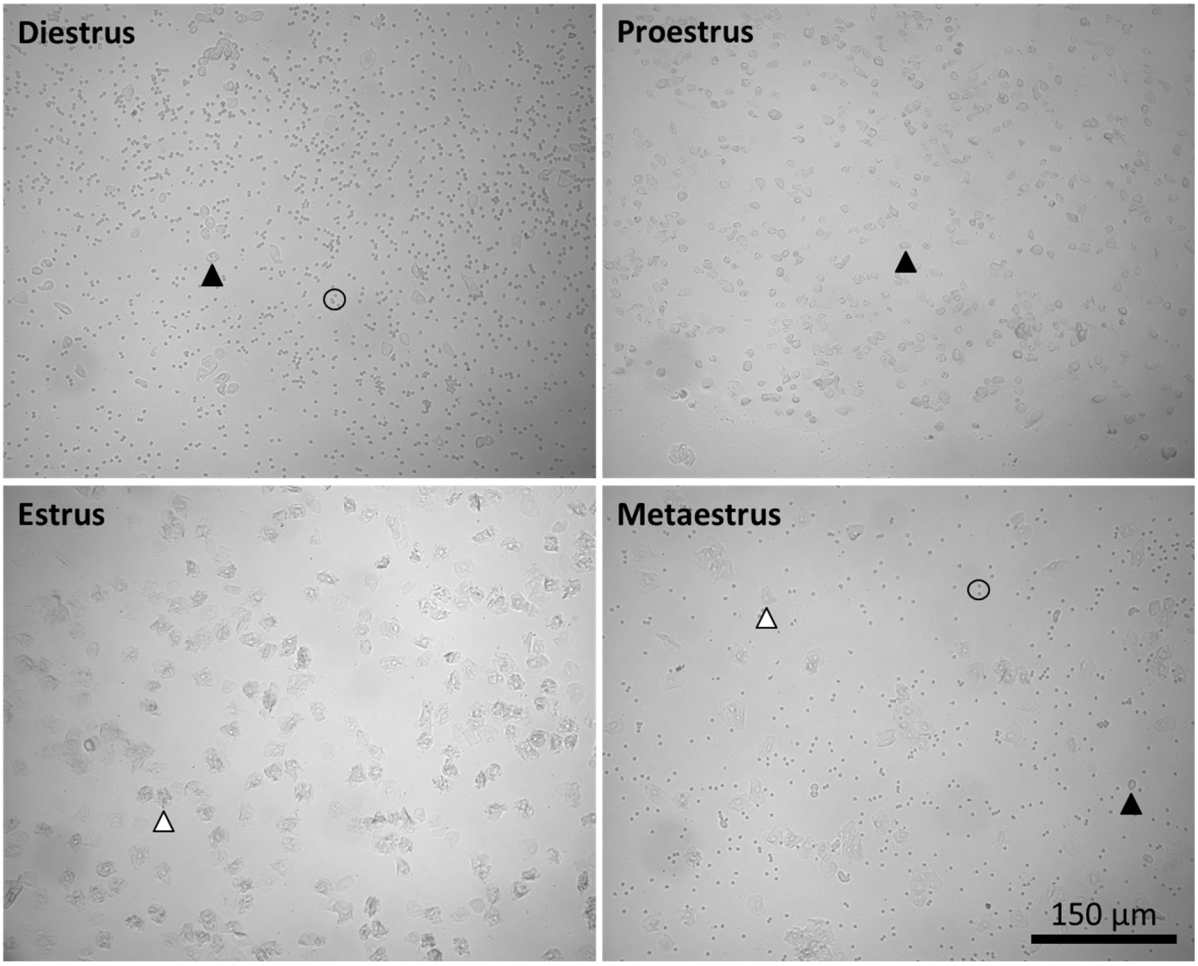

Figure 9: Determination of murine estrous cycle phase via vaginal cell cytology. Estrus phase in young CF1 mice was determined by vaginal cell cytology. Leukocytes (circle), nucleated epithelial cells (black triangle), and cornified epithelial cells (white triangle) were identified under 20X magnification.

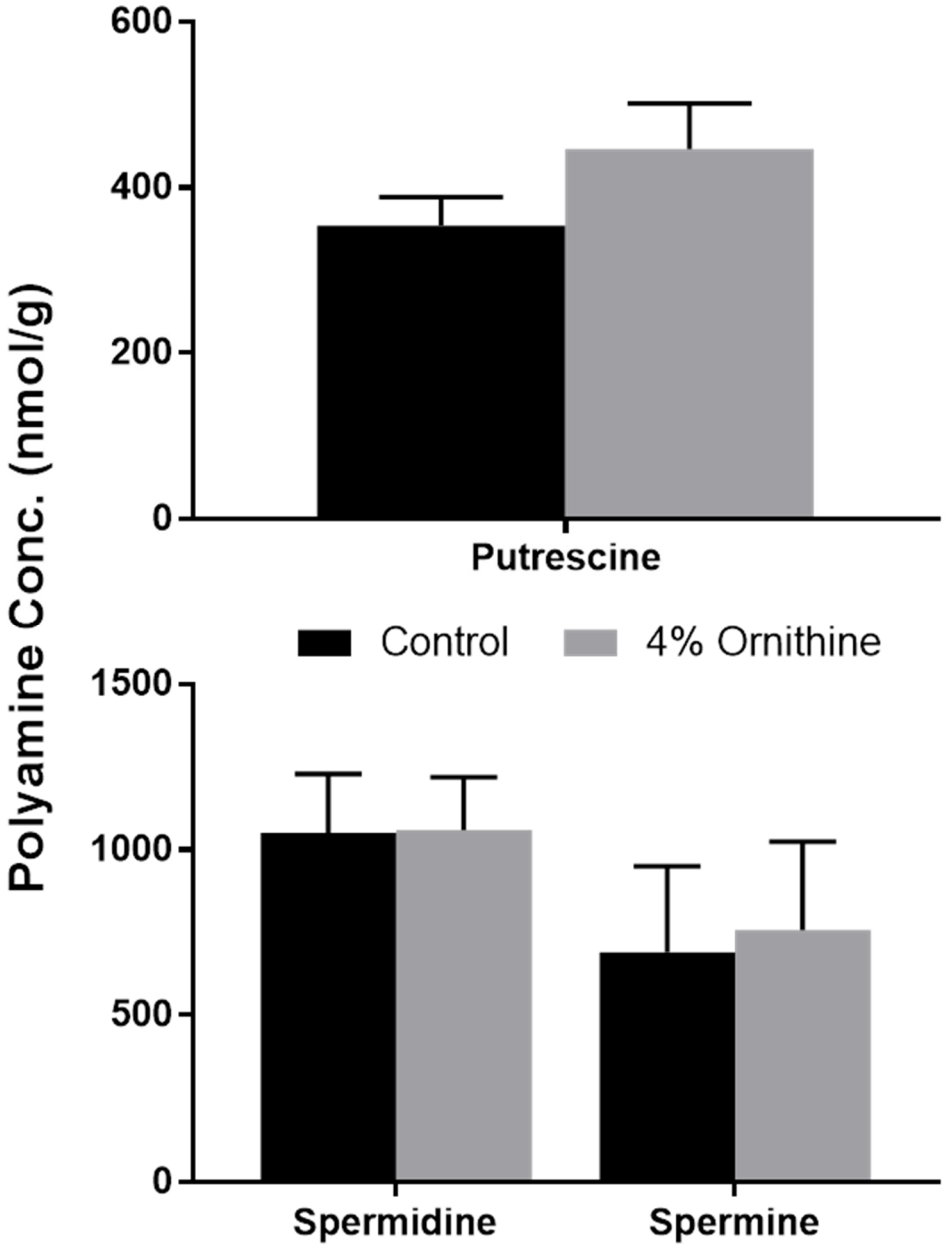

Figure 10: Ovarian polyamine concentrations in young mice treated with 4% L-ornithine in drinking water during the natural estrus cycle. Estrus cycle tracking was performed in young CF1 mice. Mice found to be in proestrus phase were separated into water (n=20) and 4% L-ornithine treated (n=23) groups. Ovarian polyamines were analysed and quantified at 02:00 the following day. Data are presented as means ± SEM.

4. Discussion

The results presented in the current study found L-ornithine can increase ovarian putrescine during the peri-ovulatory period in both young and old mice. The concentration of L-ornithine in some tissues has been analysed. Except for the liver, concentrations of L-ornithine observed in tissues tend to be below the 100 μM K_M of ODC (Coleman et al., 1993; Kitani and Fujisawa, 1988; O'Brien et al., 1986). L-ornithine is observed at an average of approximately 50 μM in the blood of mice (Takach et al., 2014) and humans (De Bandt et al., 1998; Kato et al., 1987), approximately 8 nmol/g in the brain, 32 nmol/g in the kidney, and 343 nmol/g in the liver of mice (Seiler et al., 1989). Given that L-ornithine is one of the primary intermediates in the urea cycle which, takes place in the liver, it is not surprising that high L-ornithine concentrations are observed there. As supplemental L-ornithine increases ovarian putrescine concentrations in both young and old mice, it stands to reason that the concentrations of L-ornithine in the ovary do not saturate the activity of ODC during the peri-ovulatory period. In addition, despite lower ODC activity with age (Tao and Liu, 2013), L-ornithine increased ovarian putrescine concentrations in aged mice. Moreover, the increases of ovarian putrescine concentrations were dose-dependent. Taken together, these results provide strong support that the ovarian concentration of L-ornithine does not saturate ODC activity during the peri-ovulatory period regardless of age. Unfortunately, the L-ornithine concentrations in the ovary with age have not been reported and we are unable to definitively show this given the current results. It is suggested that future work examine the concentrations of L-ornithine in the ovary and whether they are altered with age.

We also investigated the effective time of L-ornithine to increase ovarian putrescine concentrations. L-ornithine supplementation partially sustained ovarian putrescine

concentrations for several hours after administration in aged mice. This finding suggests that L-ornithine resides in the ovaries for several hours after supplementation. Although we did not analyse ovarian L-ornithine concentrations following supplementation, the concentration of L-ornithine is proportional to the amount of putrescine produced as demonstrated by our dose-dependence results. Thus, the concentrations of putrescine elicited provide insight as to the concentration of L-ornithine present in the ovary at that time allowing some conclusions to be drawn. Interestingly, the increases of ovarian putrescine concentrations following L-ornithine administration appear to be consistent with the residence time of L-ornithine in the blood reported in the literature. Peak putrescine concentrations were observed in our mice one hour after L-ornithine administration. This suggests that L-ornithine concentrations may peak in the ovary after approximately one hour. This is supported by two separate studies utilizing bolus doses of L-ornithine (150 and 170 mg/kg) in human patients finding peak blood L-ornithine concentrations approximately one hour after L-ornithine administration (Bucci et al., 1990; Kato et al., 1987). We also observed reduced putrescine concentrations with increasing time between L-ornithine supplementation and polyamine determination. L-ornithine was only found to provide increases in ovarian putrescine up to four hours after administration. By five hours after administration there is no difference in ovarian putrescine concentrations of L-ornithine treated mice compared to control mice. This is consistent with supplementation studies finding L-ornithine concentrations return to basal levels roughly four to five hours after administration (Kato et al., 1987). As the decline of serum L-ornithine concentrations and ovarian putrescine concentrations are similar, one may suggest that the clearance rate in the serum and ovaries is similar. However, the clearance rate of L-ornithine should be more clearly determined in the future to produce an effective treatment schedule should L-ornithine be utilized to increase ovarian putrescine concentrations.

Following our hypothesis and first objective, we also explored whether L-ornithine supplementation resulted in specific increases in ovarian putrescine compared to putrescine supplementation. As predicted, peri-ovulatory L-ornithine supplementation only significantly increased ovarian putrescine concentrations compared to the systemic increases caused by peri-ovulatory putrescine supplementation. This finding indicates that L-ornithine can take advantage of the LH/hCG-induced increase of ODC activity localized to the ovary (Bastida et al., 2005; Icekson et al., 1974; Tao and Liu, 2013) to specifically increase ovarian putrescine while preventing systemic putrescine increases. No tissues other than the ovaries were observed to have significantly increased putrescine concentrations compared to control mice. Although we did not examine the putrescine concentrations in all tissues, it should be a safe conclusion that L-ornithine supplementation does not result in significant increases to overall putrescine levels. Putrescine concentrations remained low in the kidney and liver indicating that no significant amount of putrescine had been or was passing through to be excreted. This supports that L-ornithine supplementation results in ovary specific increases of putrescine concentrations.

One of three L-ornithine treated mice had significantly higher putrescine concentrations in the brain, resulting in a 10-fold increase in the putrescine concentrations of the group compared to control mice. The exact cause of the increased concentration of putrescine in the brain of this mouse is unknown. However, there is evidence in the literature suggesting that ODC activity may be elevated in the brain following an ischemic event such as a stroke, coinciding with increased putrescine concentrations even several hours after the event (Dempsey et al., 1991; Henley et al., 1996). As a single mouse was responsible for the large increase of putrescine concentrations and the mice were approximately 10-months of

age, it is possible an ischemic event may have occurred. Since ODC activity and putrescine concentrations may increase many hours after an ischemic event (Dempsey et al., 1991; Henley et al., 1996), this could explain the origin of the observed increase.

In contrast to peri-ovulatory L-ornithine supplementation, peri-ovulatory putrescine supplementation induced increases of putrescine concentrations across most tissues that were examined. One of the three control mice were observed to have unusually high putrescine concentrations in the spleen, 1090 nmol/g compared to an average of 270 nmol/g for the other controls, artificially raising the mean of the control group. This was likely the result of a pathological condition. First, this was much higher than the spleen putrescine concentrations observed in any of the other aged mice in this experiment, including the putrescine treated animals. Second, this value is also much greater than the concentrations reported in the spleen of young mice (Nishimura et al., 2006). Despite this uncertainty, it seemed likely that putrescine supplementation, but not L-ornithine supplementation, increased putrescine concentrations in the spleen of aged mice (compare the spleen values of the last two groups of table I).

Since the overall mechanism for putrescine action remains unknown it may still be preferable to avoid non-specific increases of putrescine concentrations by utilizing L-ornithine. However, most literature available appears to suggest putrescine supplementation should be safe. In addition to the high LD_{50} of putrescine (Til et al., 1997), putrescine has a short half-life (Russell et al., 1970) and a very short residence time in the blood and ovaries after administration (Tao et al., 2015). Further, ODC-expressing transgenic mice with 2- to 10-fold higher putrescine concentrations in most tissues exhibit no increased risk of developing cancer or neurodegenerative diseases or have any phenotypic differences

compared to non-transgenic mice (Alhonen et al., 1995; Halmekyto et al., 1991). Moreover, excess putrescine does not affect rates of cellular proliferation (Wilson et al., 2005). Putrescine is supplemented 1% w/w in milk replacer to newborn pigs (Grant et al., 1990) and calves (Grant et al., 1989) to promote intestinal health.

The first objective we sought to explore in the current study was whether peri-ovulatory L-ornithine supplementation specifically increases ovarian putrescine concentrations in mice. We believe that we have presented strong evidence to show that L-ornithine can specifically and dose-dependently increase ovarian putrescine concentrations. It is suggested that future studies should examine ovarian L-ornithine concentrations including the effect of age on L-ornithine concentrations. In addition, alternative means of delivering L-ornithine during the peri-ovulatory period and ovulation, such as controlled release mechanisms (Priya James et al., 2014), may be sought to provide longer lasting increases in ovarian putrescine concentrations.

Despite the clear demonstration that L-ornithine administration specifically increases ovarian putrescine concentrations, our attempts to improve reproductive outcomes in aged mice via L-ornithine supplementation have been unsuccessful. One explanation for the lack of reproductive benefit may be that 1% L-ornithine supplementation generated insufficient putrescine in the ovaries. We did not determine ovarian putrescine concentrations in mice given 1% L-ornithine water. However, we could not detect significant increase of ovarian putrescine in mice given 4% L-ornithine water. In contrast, significantly higher ovarian putrescine concentrations were observed in mice given 1% putrescine water (Tao et al., 2015). Furthermore, our data indicated that the 100 mg/kg dose of L-ornithine produces lower concentrations of ovarian putrescine concentrations compared to 100 mg/kg of putrescine

(figure 5, table I). Therefore, it is likely that 1% L-ornithine supplemented in drinking water did not increase ovarian putrescine concentrations to the same extent as 1% putrescine supplementation.

In contrast to 1% L-ornithine supplementation which may have a slight reproductive benefit in aged mice, 4% L-ornithine supplementation had a clear detrimental effect. One possible reason might be the 4% L-ornithine supplementation increased ovarian putrescine concentrations to levels that became harmful or detrimental to egg quality. This appears unlikely as we did not detect significant increases of ovarian putrescine concentrations in mice supplemented with 4% L-ornithine in drinking water (figure 10). In more controlled experiments where aged mice were injected with various doses of L-ornithine or putrescine, 2.5-fold more L-ornithine than putrescine (i.e. a 250 mg/kg dose of L-ornithine vs. a 100 mg/kg dose of putrescine) was required to produce similar levels of ovarian putrescine (figure 5, table I). Therefore, in the future it may be beneficial to evaluate if an intermediate dose, for instance 2.5% L-ornithine in drinking water, can provide reproductive benefit similar to that provided by 1% putrescine supplementation in drinking water (table III, (Tao et al., 2015)).

However, 4% L-ornithine supplementation could have reduced the fertility of aged mice independently of increasing ovarian putrescine concentrations. Metabolic deficiency of OAT results in 10- to 15-fold increases of L-ornithine concentrations in almost all body fluids with modest reductions in plasma lysine, glutamine, glutamate, and creatine (Sipila, 1980; Sipila et al., 1980; Wang et al., 1995). These drastic and prolonged increases of L-ornithine concentrations result in gyrate atrophy of the choroid and retina (GA), which causes progressive vision loss and eventual blindness due to damage to the retinal pigment epithelial

cells of the eye (Simell and Takki, 1973). Despite the drastic increase in L-ornithine concentrations caused by OAT deficiency, L-ornithine supplementation is not likely to induce any detrimental effects on the eyes or vision. The safety of high dose L-ornithine supplementation has been demonstrated in animals. Treatment of rats with 4% w/w L-ornithine in food for 49 weeks caused no sign of retinal degeneration characteristic of GA (Sakamoto et al., 2015). Research has shown that L-ornithine is only toxic if concentrations are high (in excess of 600 μM) for a few years. However, constant blood L-ornithine concentrations below 600 μM do not appear to be detrimental. Moreover, transient high-dose L-ornithine supplementation was concluded to be safe for the eye (Hayasaka et al., 2011). Issues other than retinal toxicity have not been identified in the literature. Moreover, supplementation studies in humans have not indicated any dangers of L-ornithine supplementation (Chromiak and Antonio, 2002; De Bandt et al., 1998; Demura et al., 2010; Kokubo et al., 2013). However, the apparent detrimental effect of excess L-ornithine on fertility demonstrated in the current study should serve as a warning to the potential danger of excess L-ornithine supplementation for women planning pregnancy.

Two possible future approaches should be considered in pursuing L-ornithine supplementation further. First, L-ornithine can be encapsulated in a biodegradable polymer such as poly(lactic-co-glycolic acid) (Makadia and Siegel, 2011) serving as a controlled release mechanism. A controlled release L-ornithine formulation could be deposited vaginally, directly into the uterus, providing a more direct route to the ovaries. This will likely greatly reduce the amount of L-ornithine required to increase ovarian putrescine concentrations, hence minimizing the possible toxicity of L-ornithine through other pathways. The second is to examine the effect of L-ornithine supplementation in oocyte *in*

vitro maturation (IVM) media. Putrescine supplementation in IVM media can improve oocyte quality in aged mice (Liu et al., 2016a). In this approach, cumulus-oocyte complexes (COCs) are incubated in IVM media in which the oocytes are matured *in vitro* to become fertilizable eggs. Since ODC is expressed in both the oocytes and cumulus cells (Bastida et al., 2005; Icekson et al., 1974; Tao and Liu, 2013), exogenous L-ornithine present in the IVM media is expected to be converted into putrescine thereby correcting putrescine deficiency in aged COCs while avoiding the undesired effects of L-ornithine supplementation *in vivo*.

5. Conclusion

Aged animals produce significantly lower levels of ovarian putrescine during ovulation due to lower activity of ODC. Low concentrations of ovarian putrescine adversely affect the likelihood of reproductive success in aged animals. Putrescine supplementation to correct this metabolic deficiency improves reproductive success in aged animals. Herein, we have effectively shown that peri-ovulatory L-ornithine supplementation might be a viable alternative treatment. We demonstrated that administering L-ornithine specifically and dose-dependently increased ovarian putrescine in mice without changing concentrations of other polyamines in the ovaries and without altering putrescine concentrations in any other tissues examined. However, future work will be required to determine the optimal dose and route of administration to realize this potential.

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7. Appendix

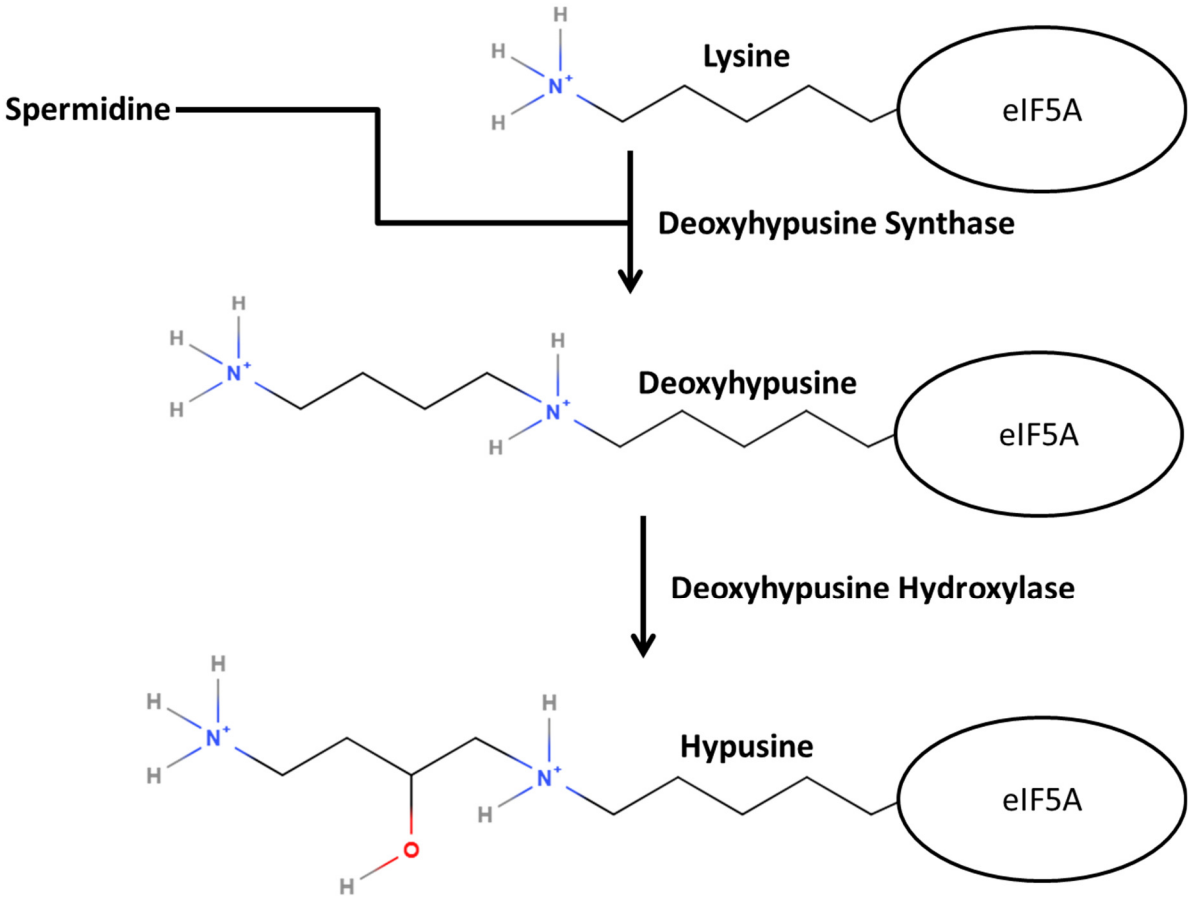

Figure 11: Conversion of lysine 50 into hypusine for the maturation of human eIF5A. Lysine 50 is converted to hypusine through the action of deoxyhypusine synthase which transfers an aminobutyl moiety from spermidine to lysine 50 to form deoxyhypusine. Deoxyhypusine hydroxylase then converts the intermediate deoxyhypusine into hypusine to form the mature and active eIF5A.

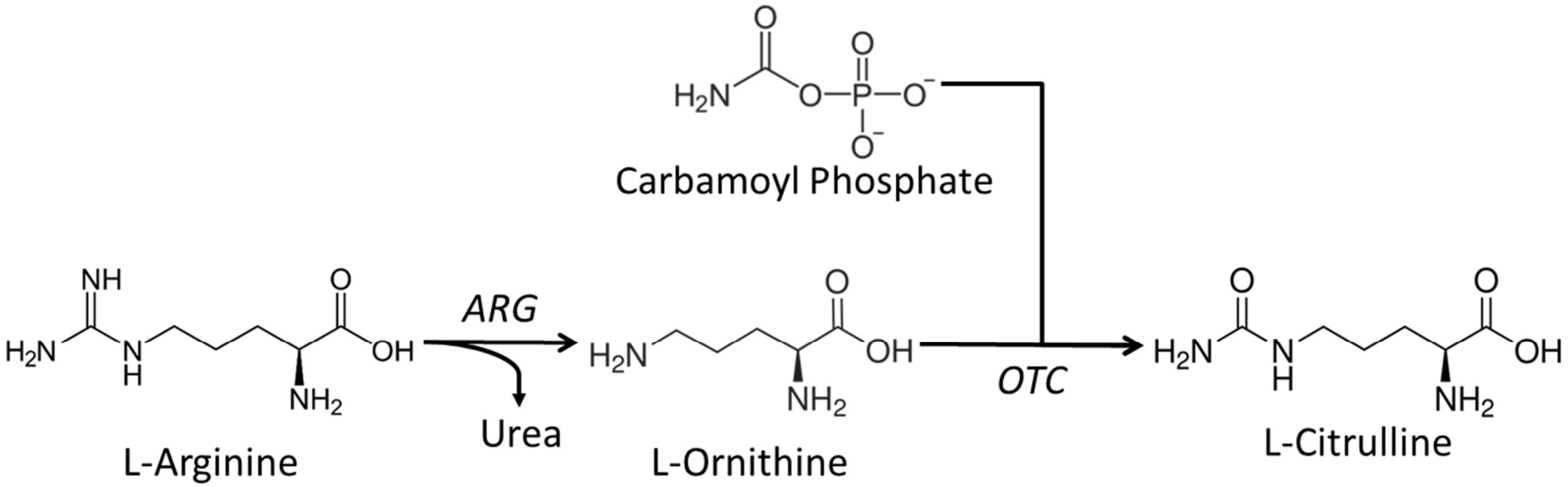

Figure 12: Production of L-ornithine and subsequent conversion to L-citrulline. L-ornithine is produced by arginase (*ARG*) with urea as a by-product. L-ornithine can then be converted to L-citrulline by ornithine transcarbamylase (*OTC*) using carbamoyl phosphate as a co-factor.

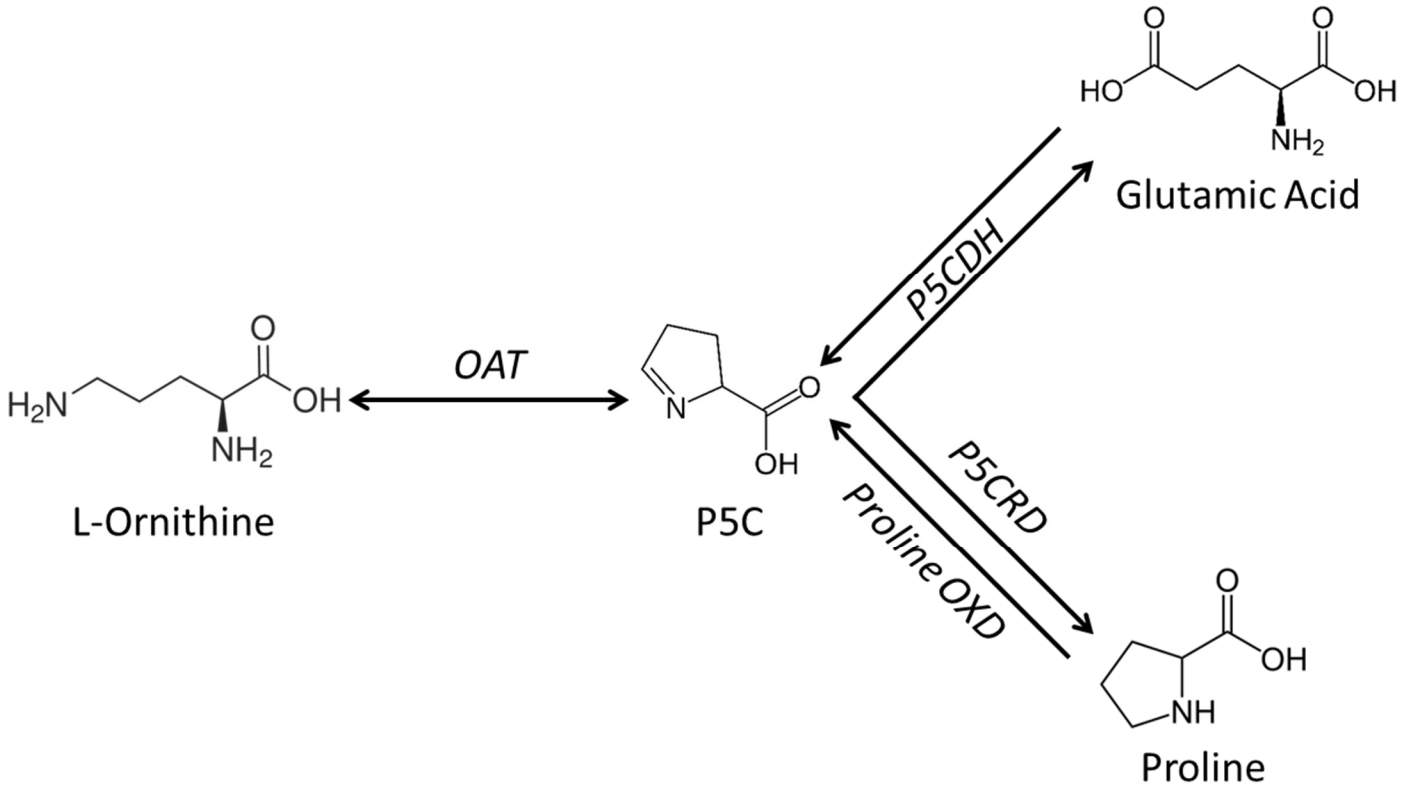

Figure 13: The reversible production of proline and glutamic acid from L-ornithine. L-ornithine can be reversely converted to pyrroline-5-carboxylate (P5C) through the action of ornithine aminotransferase (*OAT*). P5C can be converted to proline or glutamic acid by P5C reductase (*P5CRD*) and P5C dehydrogenase (*P5CDH*), respectively. Conversely, P5C may be produced from proline and glutamic acid through *P5CDH* and proline oxidase (*Proline OXD*), respectively.

Table V: Reproductive history of aged C57BL/6 female mice supplemented with 1% L-ornithine in drinking water or control drinking water.

Mouse	Age (Months)							
	9.5		11		12		13	
	Treatment	Litter Size	Treatment	Litter Size	Treatment	Litter Size	Treatment	Litter Size
126	Water	---	Water	---	Water	---	Water	---
129	Water	---	Water	---	1% ORN	---	Water	---
130	1% ORN	3	Water	---	1% ORN	---	1% ORN	---
140	1% ORN	2	1% ORN	3	1% ORN	3	Water	---
145	1% ORN	2	Water	4	Water	1	1% ORN	---
146	1% ORN	---	Water	---	1% ORN	---	Water	---
147	Water	5	1% ORN	---	1% ORN	---	1% ORN	---
149	Water	---	Water	N/A	N/A	N/A	N/A	N/A
150	1% ORN	---	Water	---	1% ORN	---	Water	---
152	Water	---	Water	---	Water	---	N/A	N/A
154	1% ORN	---	Water	4	1% ORN	---	Water	---
155	Water	---	1% ORN	---	Water	---	Water	---
157	Water	---	Water	3	Water	---	1% ORN	4
160	Water	---	1% ORN	---	1% ORN	---	Water	---
161	1% ORN	6	1% ORN	---	Water	---	N/A	N/A
164	Water	5	Water	---	1% ORN	1	N/A	N/A
165	1% ORN	---	1% ORN	---	1% ORN	---	Water	---
167	1% ORN	8	Water	---	Water	---	Water	---
170	Water	1	Water	---	Water	---	1% ORN	---
173	Water	10	1% ORN	---	Water	---	1% ORN	7
175	Water	3	Water	---	Water	---	1% ORN	---
181	1% ORN	---	Water	---	1% ORN	---	1% ORN	---
182	1% ORN	---	Water	---	Water	---	1% ORN	---
184	Water	---	1% ORN	---	Water	---	Water	2
187	1% ORN	---	Water	3	Water	6	Water	2
190	Water	---	N/A	N/A	N/A	N/A	N/A	N/A
191	Water	---	1% ORN	---	1% ORN	---	1% ORN	---
192	Water	3	Water	---	1% ORN	---	1% ORN	1
194	1% ORN	---	1% ORN	---	Water	---	1% ORN	---
195	Water	---	1% ORN	2	Water	---	1% ORN	---
196	Water	---	1% ORN	---	Water	---	Water	---
199	Water	10	1% ORN	5	1% ORN	---	1% ORN	---
200	1% ORN	5	Water	---	1% ORN	---	1% ORN	---
202	1% ORN	9	1% ORN	9	1% ORN	---	Water	---
203	Water	1	1% ORN	---	1% ORN	---	1% ORN	---

Mouse	Age (Months)							
	9.5		11		12		13	
	Treatment	Litter Size	Treatment	Litter Size	Treatment	Litter Size	Treatment	Litter Size
205	Water	---	1% ORN	3	Water	---	1% ORN	---
206	1% ORN	2	1% ORN	---	Water	---	1% ORN	---
207	1% ORN	---	Water	---	Water	---	Water	---
211	1% ORN	---	Water	---	Water	---	Water	---
212	1% ORN	---	1% ORN	6	1% ORN	2	Water	---
214	1% ORN	---	Water	1	Water	---	1% ORN	---
215	1% ORN	4	Water	---	1% ORN	---	1% ORN	---
216	Water	1	Water	1	1% ORN	---	Water	---
217	1% ORN	---	Water	---	Water	6	Water	---
219	1% ORN	7	Water	8	Water	---	1% ORN	---
220	Water	---	1% ORN	---	Water	---	Water	---
225	Water	---	Water	---	1% ORN	---	1% ORN	---
226	1% ORN	---	1% ORN	---	Water	---	Water	---
227	1% ORN	---	1% ORN	---	1% ORN	---	Water	---
228	1% ORN	4	1% ORN	---	Water	---	Water	1
229	1% ORN	---	Water	---	1% ORN	---	Water	1
230	1% ORN	---	Water	9	Water	---	1% ORN	2
231	1% ORN	1	Water	---	1% ORN	---	Water	---
232	1% ORN	---	1% ORN	---	1% ORN	6	Water	1
233	1% ORN	4	Water	---	1% ORN	---	1% ORN	---
234	1% ORN	1	1% ORN	---	Water	---	Water	---
235	1% ORN	---	1% ORN	---	Water	---	Water	---
236	Water	8	Water	---	Water	---	1% ORN	---
237	1% ORN	3	1% ORN	---	N/A	N/A	Water	---
238	1% ORN	---	1% ORN	---	Water	---	1% ORN	---
239	Water	4	Water	---	Water	---	Water	---
240	Water	---	1% ORN	---	1% ORN	---	Water	---
241	1% ORN	---	1% ORN	5	Water	---	1% ORN	---
242	Water	1	1% ORN	---	Water	---	1% ORN	---
243	Water	1	Water	---	1% ORN	---	Water	---
244	Water	4	1% ORN	---	1% ORN	---	Water	---
245	Water	---	1% ORN	6	1% ORN	---	1% ORN	---
246	Water	5	Water	---	Water	---	1% ORN	1
247	Water	---	Water	4	1% ORN	4	Water	---
248	Water	---	Water	---	1% ORN	---	Water	---
249	1% ORN	---	1% ORN	6	1% ORN	---	N/A	N/A
250	1% ORN	6	Water	---	Water	---	N/A	N/A

Mouse	Age (Months)							
	9.5		11		12		13	
	Treatment	Litter Size	Treatment	Litter Size	Treatment	Litter Size	Treatment	Litter Size
251	Water	2	1% ORN	2	Water	---	1% ORN	---
252	1% ORN	4	Water	---	1% ORN	3	Water	---
253	Water	5	Water	1	Water	---	1% ORN	---
254	1% ORN	3	Water	3	Water	---	1% ORN	---
255	1% ORN	---	Water	5	Water	4	Water	---
256	Water	1	1% ORN	4	1% ORN	2	Water	---
257	1% ORN	---	1% ORN	1	1% ORN	---	1% ORN	---
258	1% ORN	---	1% ORN	---	1% ORN	---	1% ORN	---
259	1% ORN	1	N/A	N/A	N/A	N/A	N/A	N/A
260	1% ORN	6	1% ORN	5	1% ORN	---	1% ORN	---
261	Water	---	Water	---	Water	---	1% ORN	---
262	Water	1	1% ORN	---	Water	---	1% ORN	---
263	Water	---	1% ORN	---	1% ORN	---	Water	---
264	1% ORN	5	Water	5	1% ORN	---	1% ORN	2
265	Water	---	1% ORN	---	Water	---	Water	---
266	1% ORN	---	1% ORN	1	Water	1	Water	---
267	Water	---	1% ORN	---	Water	---	1% ORN	---
268	Water	---	1% ORN	---	1% ORN	---	1% ORN	---
269	Water	---	1% ORN	5	Water	---	1% ORN	---
270	1% ORN	---	Water	---	1% ORN	---	Water	---
271	Water	1	1% ORN	---	1% ORN	---	Water	---
272	Water	8	Water	---	1% ORN	3	1% ORN	---
273	Water	6	Water	---	1% ORN	---	1% ORN	---
301	Water	---	Water	---	N/A	N/A	N/A	N/A
302	1% ORN	---	1% ORN	---	N/A	N/A	N/A	N/A
303	1% ORN	4	Water	3	N/A	N/A	N/A	N/A
304	1% ORN	4	Water	2	N/A	N/A	N/A	N/A
305	Water	5	1% ORN	4	N/A	N/A	N/A	N/A
306	Water	---	1% ORN	---	N/A	N/A	N/A	N/A
307	Water	2	1% ORN	---	N/A	N/A	N/A	N/A
308	Water	5	Water	2	N/A	N/A	N/A	N/A
309	1% ORN	---	Water	---	N/A	N/A	N/A	N/A
310	Water	6	1% ORN	7	N/A	N/A	N/A	N/A
311	Water	11	1% ORN	11	N/A	N/A	N/A	N/A
312	1% ORN	---	1% ORN	---	N/A	N/A	N/A	N/A
313	Water	---	Water	---	N/A	N/A	N/A	N/A
314	1% ORN	---	1% ORN	---	N/A	N/A	N/A	N/A

Mouse	Age (Months)							
	9.5		11		12		13	
	Treatment	Litter Size	Treatment	Litter Size	Treatment	Litter Size	Treatment	Litter Size
315	1% ORN	2	Water	1	N/A	N/A	N/A	N/A
316	1% ORN	4	1% ORN	5	N/A	N/A	N/A	N/A
317	Water	4	Water	1	N/A	N/A	N/A	N/A
318	1% ORN	---	Water	---	N/A	N/A	N/A	N/A
319	1% ORN		1% ORN	6	N/A	N/A	N/A	N/A
320	1% ORN	5	Water	---	N/A	N/A	N/A	N/A
321	Water	---	1% ORN	---	N/A	N/A	N/A	N/A
322	Water	5	Water	---	N/A	N/A	N/A	N/A
323	1% ORN	1	1% ORN	---	N/A	N/A	N/A	N/A
324	Water	3	Water	---	N/A	N/A	N/A	N/A
325	1% ORN	11	Water	---	N/A	N/A	N/A	N/A
326	1% ORN	---	1% ORN	---	N/A	N/A	N/A	N/A
327	Water	---	Water	---	N/A	N/A	N/A	N/A
328	Water	3	1% ORN	1	N/A	N/A	N/A	N/A
329	Water	4	Water	5	N/A	N/A	N/A	N/A
330	1% ORN	---	Water	---	N/A	N/A	N/A	N/A
331	Water	---	Water	5	N/A	N/A	N/A	N/A
332	Water	---	1% ORN	---	N/A	N/A	N/A	N/A
333	1% ORN	---	1% ORN	---	N/A	N/A	N/A	N/A
334	1% ORN	---	Water	---	N/A	N/A	N/A	N/A
335	Water	6	1% ORN	---	N/A	N/A	N/A	N/A
336	Water	---	Water	---	N/A	N/A	N/A	N/A
337	Water	5	1% ORN	2	N/A	N/A	N/A	N/A
338	1% ORN	---	Water	---	N/A	N/A	N/A	N/A
339	1% ORN	5	1% ORN	---	N/A	N/A	N/A	N/A
340	1% ORN	---	1% ORN	---	N/A	N/A	N/A	N/A

Table VI: Reproductive history of aged C57BL/6 female mice supplemented with 4% L-ornithine followed by 1% putrescine in drinking water or control drinking water.

Mouse	Age (Months)					
	10		11.5		12.5	
	Treatment	Litter Size	Treatment	Litter Size	Treatment	Litter Size
421	Water	6	Water	6	1% Put	8
422	Water	---	Water	---	1% Put	---
423	Water	1	Water	3	1% Put	3
424	Water	---	Water	5	1% Put	3
425	ORN	---	Water	---	1% Put	---
426	Water	---	4% ORN	---	Water	---
429	Water	---	Water	---	Water	---
432	ORN	5	Water	---	1% Put	---
433	Water	2	4% ORN	---	Water	---
434	Water	3	4% ORN	3	Water	3
436	Water	---	4% ORN	---	1% Put	---
437	Water	6	4% ORN	---	1% Put	---
439	Water	---	4% ORN	---	1% Put	---
440	ORN	---	4% ORN	---	Water	---
441	Water	6	Water	---	1% Put	---
442	ORN	4	Water	---	1% Put	2
444	ORN	2	4% ORN	---	Water	5
447	Water	---	Water	---	1% Put	---
448	ORN	---	Water	---	1% Put	---
450	Water	---	4% ORN	---	Water	---
451	Water	---	4% ORN	---	Water	---
454	ORN	---	4% ORN	---	Water	---
456	ORN	---	4% ORN	---	Water	3
457	ORN	---	4% ORN	3	Water	---
459	ORN	3	4% ORN	---	1% Put	---
460	ORN	---	Water	---	1% Put	7
461	Water	5	N/A	N/A	N/A	N/A
462	Water	---	Water	---	Water	---
463	ORN	---	Water	---	1% Put	---
466	Water	---	Water	---	Water	---
467	Water	---	4% ORN	---	Water	---
468	Water	---	4% ORN	---	1% Put	---
472	ORN	---	4% ORN	3	Water	---
473	Water	4	Water	4	1% Put	---

Mouse	Age (Months)					
	10		11.5		12.5	
	Treatment	Litter Size	Treatment	Litter Size	Treatment	Litter Size
474	Water	---	4% ORN	2	Water	---
475	ORN	1	4% ORN	---	1% Put	7
477	ORN	---	Water	---	1% Put	---
478	ORN	---	Water	---	Water	---
479	ORN	---	4% ORN	---	Water	---
481	ORN	---	4% ORN	1	Water	---
483	Water	1	4% ORN	---	Water	---
484	ORN	---	Water	3	1% Put	---
486	ORN	5	4% ORN	3	Water	---
487	ORN	---	Water	---	1% Put	---
489	ORN	3	Water	---	Water	---
490	ORN	---	Water	10	Water	---
491	Water	---	Water	---	1% Put	---
492	Water	---	Water	4	1% Put	---
493	Water	---	Water	---	Water	---
494	ORN	8	N/A	N/A	N/A	N/A
496	Water	6	4% ORN	---	Water	---
497	ORN	---	4% ORN	---	1% Put	---
498	Water	---	4% ORN	---	Water	---
499	Water	6	4% ORN	3	1% Put	---
901	Water	---	Water	---	Water	---
902	Water	---	Water	---	1% Put	---
906	ORN	---	4% ORN	---	1% Put	---
907	ORN	---	4% ORN	---	1% Put	4
908	ORN	---	Water	---	1% Put	---
910	Water	---	4% ORN	---	Water	---
911	Water	---	4% ORN	---	Water	2
913	Water	---	Water	---	1% Put	---
916	ORN	---	Water	---	Water	---
918	Water	---	Water	---	N/A	N/A

Table VII: Detailed breakdown of implantation sites at 9.5 dpc in female 10-month-old C57BL/6 mice treated with water or 4% L-ornithine in drinking water.

Mouse	Treatment	Implantations			Normal Fetuses	Resorbed Fetuses
		Left	Right	Total		
471	Water	6	6	12	10	2
912	Water	2	9	11	6	5
919	Water	5	5	10	7	3
455	Water	5	4	9	8	1
443	Water	4	5	9	7	2
485	Water	5	4	9	9	0
915	Water	3	5	8	8	0
920	Water	2	6	8	6	2
904	Water	1	5	6	2	4
480	Water	N/A	N/A	N/A	N/A	N/A
464	Water	N/A	N/A	N/A	N/A	N/A
476	Water	N/A	N/A	N/A	N/A	N/A
453	Water	N/A	N/A	N/A	N/A	N/A
458	Water	N/A	N/A	N/A	N/A	N/A
428	Water	N/A	N/A	N/A	N/A	N/A
488	Water	N/A	N/A	N/A	N/A	N/A
905	ORN	5	4	9	9	0
909	ORN	2	7	9	8	1
430	ORN	6	3	9	8	1
438	ORN	4	5	9	7	2
435	ORN	5	3	8	6	2
445	ORN	5	0	5	4	1
420	ORN	3	1	4	3	1
446	ORN	2	0	2	2	0
917	ORN	2	0	2	0	2
914	ORN	1	0	1	1	0
427	ORN	0	1	1	0	1
465	ORN	N/A	N/A	N/A	N/A	N/A
431	ORN	N/A	N/A	N/A	N/A	N/A
449	ORN	N/A	N/A	N/A	N/A	N/A
470	ORN	N/A	N/A	N/A	N/A	N/A
495	ORN	N/A	N/A	N/A	N/A	N/A
469	ORN	N/A	N/A	N/A	N/A	N/A
452	ORN	N/A	N/A	N/A	N/A	N/A
903	ORN	N/A	N/A	N/A	N/A	N/A

Curriculum Vitae

Christopher L. J. Lavergne

EDUCATION

2016 – 2018	**University of Ottawa – Ottawa, Ontario** • M.Sc. Biochemistry
2010 – 2015	**University of Ottawa – Ottawa, Ontario** • B.Sc Biology / Specialization: Cellular and Molecular Biology *Magna cum laude*
2006 – 2010	**Saint Matthew Catholic High School – Ottawa, Ontario** • Ontario Secondary School Diploma

WORKING EXPERIENCE

Jul. 2018 – Present	**Scientific Evaluator: Pest Management and Regulatory Agency, Health Canada** • Supervisor: Jennifer Selwyn • Responsible for the assessment of scientific data to determine the safety and applicability of a user-requested minor use expansion to pesticide use.
Jan. 2016 – Jul. 2018	**Master's Research** • Principal Investigator: Dr. Johné Liu (Ottawa Hospital Research Institute, Ottawa, ON) • Thesis title: Peri-Ovulatory Supplementation of L-Ornithine to Increase Reproductive Success in Aged Mice. • Exploring reproductive decline with age and attempts to rescue oocyte quality. • Recipient of Ontario Graduate Scholarship and Queen Elizabeth II Graduate Scholarship in Science and Technogy.
Apr. 2015 – Aug. 2015 & Jan. 2013 – Aug. 2013	**Laboratory Technician: Health Canada, Food Directorate, Nutritional Research Division** • Principal Investigator: Dr. Jesse Bertinato (Health Canada, Ottawa, ON) • Investigating the effect of obesity on iron requirements. • Determining the effect of increased calcium intake on nutritional biomarkers of magnesium status. • Exploring the impact of several chemical forms of magnesium on biomarkers of magnesium status. • Studying the effect of magnesium status on glucose metabolism and obesity.
May 2014 – Aug. 2014	**Pesticide Re-Evaluator: Pest Management and Regulatory Agency, Health Canada** • Supervisor: Shairoz Ramji (Health Canada, Ottawa, ON) • Responsible for re-evaluating a pesticide to determine whether or not it was safe for continued use in Canada by examining available scientific

studies and using DEEM-FCID™ to determine worst-case scenario risk to the general population from the pesticide.

Sept. 2013 – Apr. 2014 **Honours Thesis: Association of Single Nucleotide Polymorphisms (SNPs) with Biomarkers of Magnesium, Vitamin D, and Omega-3/6 Fatty Acid Status in South Asian and White Canadians.**

- Principal Investigator: Dr. Jesse Bertinato (Health Canada, Ottawa, ON)
- Examining the effect of single nucleotide polymorphisms (SNPs) on nutritional biomarkers of South Asian and White Canadians.

May 2012 –Aug. 2012 **Facility Caretaker: Animal Care and Veterinary Services (University of Ottawa, Ottawa, ON)**

- Supervisor: Bill Fletcher (Facility Manager, University of Ottawa)
- Maintained living conditions for the aquatic and amphibious animals used for research in the University of Ottawa.

VOLUNTEER EXPERIENCE

Sept. 2015 – Dec. 2015
Sept. 2014 – Apr. 2015 **Health Canada, Food Directorate, Nutritional Research Division**

- Principal Investigator: Dr. Jesse Bertinato (Health Canada, Ottawa, ON)
- Taught and assisted honors students in performing routine lab work and new lab techniques including mineral analysis, ICP-OES operation and data analyses, and running and analyzing data from ELISA assays.

LABORATORY SKILLS

BIOLOGY

- Compound and dissecting microscopy and bright field alignment
- Specimen mounting
- Dissection
- Specimen handling
- Live animal handling

BIOCHEMISTRY

- Knowledge of safe handling of various chemicals and substances
- Biochemical visualization techniques for DNA, RNA, and proteins
- ELISA assays
- DNA extraction and qPCR Analysis
- Liquid Chromatography

CHEMISTRY

- ICP-OES analyses and operation
- Mineral analysis
- Analytical weighing
- Standard curve analyses

STATISTICS

- Descriptive statistics
- One and two-way ANOVAs
- Correlation analysis
- Data analysis and presentation

TRANSFERRABLE SKILLS

WRITING AND LANGUAGE SKILLS

- Fluent English written/oral
- Scientific communication
- Technical communication

COMPUTER SKILLS

- Microsoft Office Suite
- SigmaPlot
- Soft Max Plus

- GraphPad Prism
- Database utilization (PubMed, Refworks, Endnote)

INTERPERSONAL SKILLS

- Cooperative
- Communicative
- Teaching skills

INTRAPERSONAL SKILLS

- Quick learner
- Hard working
- Resilient and perseverant
- Patient
- Independent worker

PEER REVIEWED PUBLICATIONS

Gunenc, A., Yeung, M. H., **Lavergne, C.**, Bertinato, J., & Hosseinian, F. (2017). Enhancements of antioxidant activity and mineral solubility of germinated wrinkled lentils during fermentation in kefir. *Journal of Functional Foods, 32*, 72-79. doi:http://dx.doi.org/10.1016/j.jff.2017.02.016

Bertinato, J., **Lavergne, C.**, Rahimi, S., Rachid, H., Vu, N. A., Plouffe, L. J., & Swist, E. (2016). Moderately Low Magnesium Intake Impairs Growth of Lean Body Mass in Obese-Prone and Obese-Resistant Rats Fed a High-Energy Diet. *Nutrients, 8*(5). doi:10.3390/nu8050253

Bertinato, J., **Lavergne, C.**, Vu, N. A., Plouffe, L. J., Wood, C., Griffin, P., & Xiao, C. W. (2016). l-Lysine supplementation does not affect the bioavailability of copper or iron in rats. *J Trace Elem Med Biol*. doi:10.1016/j.jtemb.2016.02.005

Bertinato, J., Wu Xiao, C., Ratnayake, W. M., Fernandez, L., **Lavergne, C.**, Wood, C., & Swist, E. (2015). Lower serum magnesium concentration is associated with diabetes, insulin resistance, and obesity in South Asian and white Canadian women but not men. *Food & nutrition research, 59*, 25974. doi:10.3402/fnr.v59.25974

Bertinato, J., Plouffe, L. J., **Lavergne, C.**, & Ly, C. (2014). Bioavailability of magnesium from inorganic and organic compounds is similar in rats fed a high phytic acid diet. *Magnesium research, 27*(4), 175-185. doi:10.1684/mrh.2014.0374

Bertinato, J., **Lavergne, C.**, Plouffe, L. J., & El Niaj, H. A. (2014). Small increases in dietary calcium above normal requirements exacerbate magnesium deficiency in rats fed a low magnesium diet. *Magnesium research, 27*(1), 35-47. doi:10.1684/mrh.2014.0360; 10.1684/mrh.2014.0360

Bertinato J., Aroche C., Plouffe LJ., Lee M., Murtaza Z., Kenney L., **Lavergne C.**, Aziz A. (2013). Diet-induced obese rats have higher iron requirements and are more vulnerable to iron deficiency. *European journal of nutrition*. doi:10.1007/s00394-013-0592-9