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UNDERSTANDING INTRINSIC AND EXTRINSIC FACTORS DRIVING INFERTILITY IN THE ENDANGERED BLACK-FOOTED FERRET (*MUSTELA NIGRIPES*)

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Dedicated to my parents as a pair for inspiring in me a true love for animals and a recognition of their depth of soul, joy, and suffering.

Dedicated to my mother, Nancy, who inspired in me the drive to use intellectual curiosity to understand how the world works and to question everything.

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Dedicated to my younger self, who would be inspired by and proud of the work I've done to contribute to endangered species conservation.

"Yesterday I was clever, so I wanted to change the world. Today I am wise, so I am changing myself."

— *Rumi*

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CHAPTER ONE | General Introduction

1.1 | Decreasing wildlife populations

The era of the Anthropocene is characterized by the disproportionate negative impact that humans are having on the environment, including devastating effects on wildlife species (Sandra et al., 2019). Recent evidence showed that one million plant and animal species were at risk of extinction due to anthropogenic changes in the land and sea (Brondizio et al., 2019). This heavy modification of natural habitats reduces population sizes, necessitating human intervention to save species (IUCN SSC, 2014).

1.2 | Wildlife in captivity

Oftentimes, species are brought into captivity at the point in which the population is so heavily reduced it is in danger of not being able to recover (Leberg & Firmin, 2008). These small populations that are subsequently captively managed pose the serious issue of low genetic diversity (Ralls & Ballou, 1986). Small population sizes lead to increased instances of inbreeding, where two closely related individuals mate (Frankham, 1995). This leads to an increase in homozygosity, which reduces fitness by increasing the likelihood that recessive alleles will be expressed (Charlesworth & Charlesworth, 1987; Roff, 2002). Reduced heterozygosity has been correlated with a decrease in fecundity, higher juvenile mortality, spermatozoa (sperm) abnormality, and even increased adult mortality (Comizzoli et al., 2010; Fitzpatrick & Evans, 2009; Losdat et al., 2014; Ralls & Ballou, 1982; Ryan et al., 2002; van Noordwijk & Scharloo, 1981). This cascade of trait value reduction is termed inbreeding depression (Charlesworth et al., 1987; Okada et al., 2011).

1.3 | Environmental-dependent inbreeding depression

Inbreeding depression has variable effects under differing circumstances (Fox & Reed, 2011). Environmental-dependent inbreeding depression (EDID) theory posits that only under certain environments will deleterious mutations cause a serious impact on fitness; i.e. inbreeding depression has variable magnitudes based on what environmental condition is encountered (Cheptou & Donohue, 2011; Szulkin & Sheldon, 2007). In other words, deleterious mutations may not negatively impact an organism if the environment does not exacerbate it. Studies have confirmed that inbreeding depression is exacerbated under stressful conditions (Cheptou et al., 2011; Dahlgaard & Hoffmann, 2000).

1.4 | Black-footed ferret

The endangered black-footed ferret (ferret; *Mustela nigripes*) may be experiencing EDID. This species experienced a genetic bottleneck as its range became diminished when European settlers spread across North America in the early 1900s (Wisely et al., 2002). During this time, the Great Plains was converted for agriculture and urban development and the ferret's habitat and its main prey species, prairie dog (*Cynomys* spp.) populations were decimated (Miller et al., 1996). As a result, the ferret was brought to near-extinction. In the 1980s, the United States Fish and Wildlife Service (USFWS) brought the last 18 remaining ferrets into captivity in an effort to save the species via an *ex-situ* breeding and reintroduction program (Miller et al., 1996). Seven individuals became the founders for the entire population that exists today.

After more than 30 years of breeding, captive ferret fertility rate has plummeted, with whelping rates dropping from 60% in the 1990s to 46% in 2021 (Marinari, 2021). The percent of morphologically normal sperm (NSM) in an ejaculate is highly correlated with fertility (Brugh &

Lipshultz, 2004; N. Kumar & Singh, 2015; Pukazhenthi et al., 2006), and the decline in ferret whelping rates correlate with a decline in NSM (Santymire et al., 2019). Specifically, in captive males, NSM has declined from 50% in 1990 to 35% in 2021 (Santymire et al. 2019; Santymire, pers. comm.).

While captive male ferrets are experiencing a steady decline in NSM, wild ferrets, who stem from the same gene pool as captives, were found to have nearly double (57.5%) the percent normal sperm than captive ferrets in 2020 (Santymire, pers. comm.). While the populations of both captive and wild ferrets are genetically similar (Wisely et al., 2002), it seems that the *ex situ* environment may be negatively affecting ferret reproductive health parameters and success (Santymire et al., 2021). This is a direct argument for EDID taking place in the ferret.

1.5 | How the ferret diet potentially generates harmful, oxidative conditions

One major difference between captive and wild ferrets is their diet. Wild ferrets eat prairie dogs along with other small rodents (Brickner et al., 2014), whereas captive ferrets are fed a standardized, commercially-produced small carnivore diet (TOR; Milliken Meats Products, Lid, Markham, Ontario, Canada) along with whole carcass (usually rat, mouse or hamster). A decline in captive male ejaculate health began the year after all ferrets were switched to this TOR diet. Previously, their diet was composed of 60% mink pellets and 40% rabbit meat and made at each facility individually before it was decided that a standardized diet should be implemented across the SSP (Santymire et al., 2004).

1.6 | Toxic vitamin A, reactive oxygen species & oxidative stress

The TOR diet is high in vitamin A (R M Santymire et al., 2015), which may be inducing oxidative stress in the ferrets and resulting in damaged sperm. At high doses, vitamin A is toxic and has been shown to become pro-oxidative and also suppress antioxidant activity by antagonizing vitamin E (Agarwal & Saleh, 2002; de Oliveira et al., 2009). This can lead to oxidative stress, cellular damage caused by an excess of reactive oxygen species (ROS) and a dearth of antioxidants (Tunc & Tremellen, 2009). ROS is a collective term for free radicals (molecules containing unpaired electrons) and nonradicals that are highly reactive oxygen molecules (C. Wright et al., 2014). ROS and specifically free radicals can act indiscriminately by accepting an electron from other molecules, thereby "oxidizing" them (R. Li et al., 2016).

ROS are byproducts of aerobic metabolism and essential to many biological and cellular pathways (C. Wright et al., 2014). Sperm itself produces ROS, particularly via the mitochondria, which powers the tail of the sperm (De Iuliis & Aitken, 2009). Low levels of ROS are also critical for sperm capacitation, stimulating the acrosome reaction which allows the sperm to bind to and digest through the egg, as well as sperm hyperactivation, the process of ultra-high motility that allows the sperm cell to penetrate the egg (Tremellen, 2008).

Additionally, leukocytes found in semen produce up to 1,000 times more ROS than sperm cells (Henkel, 2011). This high production of ROS ensures the role of leukocytes as defenders from infection and inflammation (Henkel, 2011). While biologically critical, excess amounts of ROS caused by endogenous generation coupled with exogenous sources coming from the environment, can overwhelm a system and cause oxidative damage when not balanced by antioxidants (Harlev et al., 2017).

1.7 | Oxidative damage in sperm cells

My Ph.D. explores the hypothesis that the excess vitamin A in the TOR diet is suppressing antioxidant activity in the ferret, thereby leading to oxidative stress and thus reduced fertility outcomes (Agarwal et al., 2002). We suggest the mechanism of oxidative damage on sperm cells to be two-fold. Firstly, male germ cells are particularly sensitive to oxidative stress (De Iuliis et al., 2009; Lewis & Aitken, 2005). The cell membranes of spermatozoa are composed largely polyunsaturated fatty acids which create the fluidity that allows fusion during fertilization (De Iuliis et al., 2009). However, the double bonds of the unsaturated fatty acids are vulnerable to attack by free radicals (ROS that contain an unpaired electron), which create lipid peroxide radicals (C. Wright et al., 2014). This reaction cascades by reacting with neighboring molecules in the membrane, leading to lipid peroxidation, which causes the membrane to be weakened, altering morphology, and can lead to infertility (Henkel, 2011). The decline in NSM we see in ferrets may be due to lipid peroxidation caused by oxidative stress from excess vitamin A in the TOR diet.

Further, oxidative stress can cause damage to DNA in cells (Wright et al., 2014). Most commonly, free radicals can nick the pyrimidine and purine bases as well as the deoxyribose backbone of DNA, thereby causing fragmentation (Tremellen, 2008). Additionally, oxidative stress can trigger sperm cell apoptosis which leads to caspase-activated DNA degradation (Villegas et al., 2005). In sperm cells, DNA fragmentation is highly correlated with abnormal sperm parameters such as reduced motility, concentration, and morphology (Lewis et al., 2005; Velez de la Calle et al., 2008). Sperm DNA fragmentation is also associated with decreased fertility (Gosálvez et al., 2014; Lewis et al., 2005). Several studies have shown that DNA fragmentation is negatively correlated with chance of natural or assisted pregnancy, and also

leads to spontaneous abortion (Kumar et al., 2012; Zini, 2011). In additional to abnormal sperm parameters, the decline in ferret whelping rates may also be due to oxidative damage to DNA, leading to fragmentation in sperm.

1.8 | My dissertation research

At the core of my work is the understanding that the increase in inbreeding is a central driver in the symptoms of infertility that we see. Fundamentally, what is happening in the ferret is inbreeding depression. However, given that the symptoms of infertility manifest differently between captively managed ferrets and wild ferrets (Ali et al. 2022, in prep; Santymire, Livieri personal comm.), this presents an interesting opportunity to further explore why the magnitude of inbreeding depression seems to be different across these environments. In fact, wild ferrets may be even more inbred than captive ferrets, since captive ferrets are rigorously managed using specialized software to minimize inbreeding coefficients across mated pairs, whereas wild ferrets may mate at random (Reed et al., 2002). However, wild ferrets still have better sperm parameters and higher fertility rates than captive ferrets (Santymire, personal comm.).

The variability in fertility between wild and captive ferrets supports the theory that EDID is taking place. And if EDID can mean that wild ferrets are able to have improved fertility over their ancestral, source population, which is even more rigorously managed genetically, then this begs the question as to whether conditions can be recreated in captivity to achieve similar outcomes for captive ferrets. While many conditions for a captively managed endangered species are difficult to change due to government regulations, space constraints, standardization policies, etc., diet is an obvious and manageable part of the ferret's everyday life that also has a direct

impact on overall and reproductive health. Altering the diet and creating experimental conditions across diets allows researchers a way to explore EDID.

My Ph.D. research largely explores EDID by focusing on whether and how the environment (i.e. diet) influences metrics of fertility. My work explores whether there exists evidence for oxidative stress in the ferrets due to differences in diet, and whether this impacts fertility metrics such as sperm morphology, sperm DNA integrity, and reproductive outcome (Chapter Two). In order to determine whether there exist gene expression patterns that drive some of the symptoms of infertility that we observe, and also to see whether we observe signatures of repair mechanisms that might be active and driven by particular RNAs across certain diets, I explore the ferret sperm transcriptome (Chapter Three). This is one of only a few studies that explores the sperm transcriptome in a non-model organism and endangered species, and is a de novo transcript assembly (Ran et al., 2018; Schuster et al., 2016). I also explore the potential for existing, routinely collected data to be a driver for understanding what factors impact the ability to predict whether a particular mating pair will successfully reproduce (Chapter Four). Pinpointing which factors most contribute to the cases of successful reproduction can help managers focus recovery on certain aspects of ferret health or environment.

The results of my work support the idea that certain conditions can be emulated in captivity to create an opportunity to improve ferret reproductive health. Specifically, we demonstrate that nutrition, either during early development or transgenerationally, significantly impacts metrics that contribute to fertility (i.e. oxidative stress levels and sperm DNA damage levels). While we did not observe improvements in reproductive outcomes of one diet group over another, the long-term implications of improved metrics can contribute to an eventual shift in higher whelping rates in subsequent generations.

Further, my work helps to elucidate the gene expression patterns that we see across captive and wild ferrets and across diet treatments. We find evidence of superior sperm metrics in wild ferrets from the transcriptome level, validating the importance of environment on gene expression and how it has long-term phenotypic consequences. We also find upregulation across different diets in genes that may help with DNA repair, oxidative repair pathway activation, and sperm functions related to fertility, all which help characterize the ferret sperm transcriptome, which has never before been done.

Lastly, my work successfully employs the use of machine learning methodologies, which are not commonly used in conservation work, to predict reproductive outcomes using routinely collected data. The implications of this work are that existing data from captive breeding programs can be used in creative ways to gain insight about potential management changes that are needed to improve chances at reproductive success.

CHAPTER TWO | Supplementing vitamin E over generations reduces damage from oxidative stress in black-footed ferrets

2.1 | Abstract

An unbalanced diet can generate oxidative stress, cellular oxidative damage due to an excess of reactive oxygen species (ROS) in a system and a dearth of antioxidants. Oxidative stress has been shown to significantly impact sperm health, which can thereby impact fertility. Captive black-footed ferrets saw a decline in normal sperm parameters when they were switched to a diet high in vitamin A, which is antagonistic to antioxidants and therefore, may induce oxidative stress. We tested whether a vitamin E-supplemented diet would increase antioxidant levels and therefore, reduce oxidative stress and symptoms of oxidative damage, such as reduction of damaged sperm morphologies or sperm DNA damage. We found that circulating vitamin E levels did increase in ferrets who were switched to a supplemented diet, but that overall, there was no significant difference in vitamin E or A levels across all diet treatments. Second-generation vitamin E ferrets, whose mothers were on the antioxidant supplement during conception and pregnancy, had reduced levels of DNA damage. We also found that total antioxidant capacity, a proxy for the ability for antioxidant to ward off oxidative stress, was lower in second-generation vitamin E ferrets, indicating their demand for oxidative repair was lower. Further, we found that wild ferrets had significantly lower levels of damaged acrosome and apical ridge, an indication of their superior ability for fertilization capacity. Despite there being no significant difference between reproductive status across diets, these results underscore the ability for diet to influence parameters that impact fertility, which may have implications for the reproductive health and success of future generations.

2.2 | Introduction

The era of the Anthropocene is characterized by the disproportionate negative impact that humans are having on the environment, including devastating effects on wildlife species. Recent evidence showed that one million plant and animal species were at risk of extinction due to anthropogenic changes in the land and sea (Brondizio et al., 2019). This heavy modification of natural habitats reduces population sizes, necessitating human intervention to save species (IUCN SSC, 2014).

Oftentimes, species are brought into captivity at the point in which the population is so heavily reduced it is in danger of not being able to recover. These small populations that are subsequently captively managed pose the serious issue of low genetic diversity (Che-Castaldo et al., 2019). Small population sizes lead to increased instances of inbreeding, where two closely related individuals mate. This leads to an increase in homozygosity, which reduces fitness by increasing the likelihood that recessive alleles will be expressed (Charlesworth et al., 1987; Roff, 2002). Reduced heterozygosity has been correlated with a decrease in fecundity, higher juvenile mortality, spermatozoa (sperm) abnormality, and even increased adult mortality (Comizzoli et al., 2010; Fitzpatrick et al., 2009; Losdat et al., 2014; Ralls et al., 1982; Ryan et al., 2002; van Noordwijk et al., 1981). This cascade of trait value reduction is termed inbreeding depression (Charlesworth et al., 1987; Okada et al., 2011).

Inbreeding depression can have variable effects under differing circumstances (Fox et al., 2011). Environmental-dependent inbreeding depression (EDID) theory posits that only under certain environments will deleterious mutations cause a serious impact on fitness. In other words, deleterious mutations may not negatively impact an organism if the environment does not

exacerbate it. Inbreeding depression has variable magnitudes based on what environmental condition is encountered (Cheptou et al., 2011; Szulkin et al., 2007).

Santymire and colleagues suggested that the endangered black-footed ferret (ferret; *Mustela nigripes*) is experiencing EDID (2021). This species experienced a genetic bottleneck as its range became diminished when European settlers spread across North America in the early 1900s (Wisely et al., 2002). During this time, the Great Plains was converted for agriculture and urban development and the ferret's habitat and populations of its main prey, prairie dogs (*Cynomys* spp.), were decimated (Miller et al., 1996). As a result, the ferret was brought to near-extinction. In the 1980s, the United States Fish and Wildlife Service (USFWS) brought the last 18 remaining ferrets into captivity in an effort to save the species via an *ex-situ* breeding and reintroduction program (Miller et al., 1996). Seven individuals became the founders for the entire population that exists today. After more than 30 years of breeding, captive ferret fertility rate has plummeted, with pregnancy rates dropping from 60% in the 1990s to 46% in 2021 (Marinari, 2021).

While captive male ferrets are experiencing a steady decline in normal sperm quality, dropping from 50% in 1990 to 35% in 2021 (Santymire et al.2019; Santymire, pers. Comm.), wild ferrets, who stem from the same gene pool as captives, had nearly double (57.5%) the percent normal sperm in 2020 (Santymire, pers. Comm.). The percent of morphologically normal sperm in an ejaculate is highly correlated with fertility (Brugh et al., 2004; N. Kumar et al., 2015; Pukazhenthi et al., 2006), and the decline in ferret whelping rates correlates with this decline in normal sperm (Santymire et al., 2019). While the populations of both captive and wild ferrets are genetically similar (Wisely et al., 2002), it seems that the *ex situ* environment may be negatively affecting ferret reproductive success as they become increasingly inbred (Santymire et al., 2021).

One major difference between captive and wild ferrets is their diet. Wild ferrets eat prairie dogs along with other small rodents (Brickner et al., 2014), whereas captive ferrets are fed a standardized, commercially-produced small carnivore diet (TOR; Milliken Meats Products, Lid, Markham, Ontario, Canada) along with whole carcass (usually rat, mouse or hamster). A decline in captive male ejaculate health began the year after all ferrets were switched to this TOR diet. Previously, their diet was composed of 60% mink pellets and 40% rabbit meat and made at each facility individually before a standardized diet was implemented across the SSP (Santymire et al., 2004).

The TOR diet is composed of horse meat and is high in polyunsaturated fatty acids (PUFAs) and vitamin A. High levels of vitamin A can prevent antioxidant activity, which can lead to oxidative stress (Agarwal et al., 2002). Oxidative stress occurs when reactive oxygen species (ROS) overwhelm the antioxidant defense mechanisms in a system (Sies et al., 2017). ROS are oxidative radicals that are byproducts of metabolism and can also be exogenously introduced, and are essential to many biological and cellular pathways (Thomas, 2000; Tremellen, 2008; C. Wright et al., 2014). If a system cannot balance out ROS through antioxidant activity, this can lead to oxidative damage. For example, sperm cell membranes, which are composed largely of PUFAs, are vulnerable to lipid peroxidation, which is a cascade of oxidative attacks on the lipids that make up the membrane. Additionally, if a diet is high in PUFAs, sperm PUFA concentration can increase (Van Tran et al., 2017), making sperm even more susceptible to damage in the presence of oxidative stress. This can impact sperm morphology, sperm viability, sperm motility, DNA integrity and thereby fertility (Agarwal et al., 2002).

Further, studies have shown that oxidative stress can cause DNA damage in sperm cells (De Iuliis et al., 2009; Tremellen, 2008). Oxidative stress causes DNA base pair damage as well as strand breaks (Sies, 2007; Wallace, 2002). DNA fragmentation is also associated with decreased fertility and blastocyst developmental issues (Gosálvez et al., 2014; Lewis et al., 2005; Tremellen, 2008). DNA damage also correlates with inbreeding. Ruiz-Lopez et al. (2010) found that DNA fragmentation significantly increased with inbreeding in three endangered gazelle species (*Gazella cuvieri, Gazella dama mhorr, and Gazella dorcas neglecta*). Petrovic et al. (2013) found that inbred rams had a higher incidence of DNA damage than a control group. While DNA fragmentation in sperm is highly correlated with abnormal sperm parameters such as reduced motility, concentration, and morphology (Lewis et al., 2005; Velez de la Calle et al., 2008), it has not yet been investigated in the black-footed ferret.

Numerous studies have found that supplementing vitamin E to diet can improve semen quality, since antioxidants neutralize attack by ROS (Contri et al., 2011; Yue et al., 2010). The antioxidant defense system is composed of enzymes, proteins, trace elements, micronutrients and vitamins (Sies, 2007). Some studies show that while antioxidants may not improve basic sperm parameters, they reduce DNA damage (Majzoub et al., 2017; Ross et al., 2010). Vitamin E and C supplementation for two months significantly improved DNA damage in infertile men, but did not improve most other sperm parameters (Greco et al., 2005).

The effects of supplementing the TOR diet with vitamin E and/or whole carcass was evaluated on male ferret reproduction previously (Santymire et al., 2015; 2020). Investigators found that vitamin E supplementation to a control diet did not decrease vitamin A levels and did not improve sperm parameters. However, this research only investigated the effect of the dietary supplementation over one breeding season; we were interested in examining the effects over

several generations, and a longer treatment period of time may increase chances for the detection of effects. Additionally, DNA damage changes were not assessed by Santymire et al (2015, 2020). We also collected samples from offspring, to elucidate whether potential changes in the germ line are passed to offspring and impact their reproductive health.

We hypothesized that diet significantly impacts reproductive health in the ferret. We predicted that the vitamin E and carcass treatments would significantly increase the ferret's antioxidative capacity, leading to improved sperm parameters and lower DNA damage results as compared with the control group. To test our hypothesis, we initiated a study that would evaluate three diet treatments: the control diet (TOR + two prey items per week), the control diet supplemented with vitamin E, and a prey-only diet that simulates a wild diet, over three breeding seasons. Our specific objective was to assess the effects of the diet treatments on sperm parameters, including DNA damage levels, as well as total antioxidative capacity (TAC) in serum and seminal plasma and serum vitamin levels E and A levels.

2.3 | Methods

2.3.1 | Dietary treatments

This study took place over three breeding seasons (2017-2021; 2020 is missing due to COVID-19). At the USFWS National Black-Footed Ferret Conservation Center (FCC) in Carr, Colorado, males and females were divided into two treatments: 1) TOR + prey item (2 per week; rat or hamster pieces), which served as the control; and 2) TOR + vitamin E (D-α-tocopherol; 400 IU/kg dry matter basis) + prey item (2 per week). At Louisville Zoological Gardens (LZG; Louisville, KY), a smaller population of 14 males and 16 females was divided into two treatments: 1) TOR + prey item (two rats or mice pieces/week), which served as the control; 2)

prey only (carcass; rat or mice). This treatment was to emulate the wild diet. This study was approved the Lincoln Park Zoo Research Committee (proposal #2007–005) and IACUC (2007-005) and United States Fish and Wildlife Service (Carr, CO).

Supplementation of vitamin E and/or feeding carcass-only was initiated in December 2017. These individuals were already adults and we considered them to be the first-generation vitamin E (first-gen vitamin E) or carcass individuals. Ferrets born to first-gen vitamin E diet ferrets were kept on vitamin E diets as well and were considered the second-generation vitamin E (second-gen vitamin E) or carcass individuals. Finally, ferrets that were originally on a control treatment and stayed on the control diet along with their offspring were controls. When "diet" is referred to throughout the analyses, it includes the generation component for vitamin E-supplemented individuals.

2.3.2 | Breeding Data

Reproductive data were collected by managers at the two facilities and were subsequently combined and standardized in Python (Version 3, Van Rossum & Drake, 2009). Breeding records spanned 2004 until 2020, but were matched for the three years of the diet study (2017-2021). Not all individuals were paired for reproduction and therefore, not all records have an associated breeding outcome.

2.3.3 | Sample collection, processing & storage

For males, testes firmness is an indicator of sperm production and breeding readiness (E. S. Williams et al., 1991). Semen was collected from anesthetized males (Ketaject 38.8 mg/kg; diazepam 0.06 mg/kg) by electroejaculation (Howard, 1993) in March and April depending on

testicular tumescence via palpation (R M Santymire et al., 2006, 2007). A rectal probe delivered electro-stimulations over a voltage range of 2-5, and semen was collected using a micropipette (Howard, 1993; Wolf et al., 2000). After semen collection, cells were evaluated for percent motility, sperm forward progression (motility status of 0-5; 5 is the highest score), morphology metrics (including presence of droplets, bent tail) and concentration; acrosomal integrity was assessed using phase-contrast microscopy (1000×) on 100 sperm cells per sample, as described previously (R M Santymire et al., 2006; Wildt et al., 1989).

At least 1 µL of semen was collected in 100 uL of Test Yolk Buffer (TYB; Irvine Scientific), a semen extender, for DNA fragmentation analysis via the TUNEL assay (see methods below). DNA fragmentation samples had an additional 100 µl of TYB 8% glycerol (Irvine Scientific) cryoprotectant added to bring the sample concentration to 4% TYB. After equilibrating for 10 additional minutes, the sample was stored in -80ºC.

An additional 1 µL of semen was collected in 100 µL of phosphate buffered saline (PBS) for antioxidant analysis via the Total Antioxidant Capacity (TAC) assay. Sperm cells were pelleted via centrifugation (5 minutes at 100 x*g*), and the seminal plasma was pipetted off and saved for later TAC analysis (see methods below). All samples were slow cooled for 30 minutes at 5ºC. After this period, TAC samples were stored at -80ºC.

Blood samples were collected each year of the study by a veterinarian on anesthetized (via isoflurane) ferrets during physical exams. Blood samples were placed in serum separation vials and centrifuged (20 min at 1500 rpm). 1 mL of serum was aliquoted for future vitamin analysis, and up to 1mL aliquoted for future TAC analysis. Serum was then stored at -80ºC.

Wild black-footed ferrets (n = 4 males) were all wild-born and trapped in March 2020 at Rocky Mountain Arsenal, Colorado. Black-footed ferret reintroduction at this site began in 2015.

Trapping and immobilization followed protocols of the Black-Footed Ferret Recovery Implementation Team (USFWS, 2016) and Kreeger et al. (1998). Briefly, animals were cage-trapped at night and returned to the same location following examination and recovery from anesthesia, usually within 1 hr of capture. All trapping was authorized by the USFWS under permit #TE064682-1 and was conducted by the U.S. Forest Service, National Park Service and Prairie Wildlife Research as part of routine population monitoring. All animal experiments conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Lincoln Park Zoo Research Committee (Chicago, IL) and USFWS (Carr, CO). Semen and blood sample collection followed the same procedures as above.

2.3.4 | Vitamin analysis

Frozen blood serum samples were shipped to Colorado State University's Veterinarian Diagnostic Lab (Fort Collins, CO) for vitamin analysis. The lab quantified vitamin A and E levels in μg/mL detected in the samples using liquid chromatography.

2.3.5 | Total Antioxidant Capacity (TAC) analysis

Measuring TAC is a technique used to estimate antioxidant capacity globally instead of having to measure each antioxidant separately (Rubio et al., 2016). In this study, TAC can be used as a proxy for oxidative stress. The TAC Assay Kit (MAK187 SIGMA) measures the ability of the sample to reduce Cu^{2+} to Cu^{1+}; copper is an oxidant. By measuring the absorbency of the reduced oxidizing complex, the kit provides an indirect way to measure the antioxidant ability of the sample (Sigma-Aldrich Technical Bulletin).

Seminal plasma and blood serum were analyzed for TAC using the manufacturer's instructions. The trolox standard was reconstituted with 20 µl of DMSO, vortexed, and 980 µl of milliQ water was added to make a 1mM trolox standard solution. The following standards (nmole/well) were prepared and brought up to 100ul in milliQ water: 20, 16, 12, 8, 4, 3, 2, 1, 0. The Cu^{2+} working solution was prepared as a 1:50 dilution with milliQ water. Plasma samples were diluted 1:500. Seminal fluid samples were diluted 1:4. Standards and 100 µl of diluted sample were pipetted onto the 96 well plate, and to all wells 100 µl of Cu^{2+} working solution was added. Plates were shaken briefly on a horizontal shaker and incubated at room temperature in the dark for 90 minutes. Absorbance was read at 570nm on Softmax Pro 7 software. Means of duplicates were taken, coefficient of variations were calculated and the final result was multiplied by 500 (the dilution factor). Final values were expressed in Trolox equivalents (nmol/well).

2.3.6 | Sperm DNA Damage Analysis

Semen samples collected in TYB were washed in 1 mL of phosphate buffered saline (PBS) and centrifuged for 5 minutes at 100x*g*. The supernatant was removed. This process was repeated three times to ensure removal of TYB. Then, the manufacturer's instructions were followed using the Apo-Direct Kit (Invitrogen). Cells were suspended in 5 mL of 1% paraformaldehyde and incubated on ice for 15 minutes. They were then centrifuged for 5 minutes at 1000rpm, and the wash and centrifugation were repeated. Cells were then resuspended in 0.5 mL of PBS and then resuspended in ice cold 70% ETOH on ice for 30 minutes. Positive and negative control cells (somatic) were provided in the kit and suspended in 70% ETOH. One mL of the controls was centrifuged for 5 minutes at 1000 rpm and the alcohol aspirated, leaving the

pellet undisturbed. Control cells and samples were resuspended in 1 mL of wash buffer, centrifuged as previously described, and had supernatant removed by aspiration. Each sample was then resuspended in 50 µl of the kit's DNA labeling solution. The cells were incubated overnight in the dark at room temperature. The next day, 1 mL of rinse buffer was added to each sample, samples were centrifuged as before and supernatant was aspirated off. Cells were resuspended in 0.5 mL of propidium iodide and incubated at room temperature for a minimum of 30 minutes. Cells were then analyzed by flow cytometry on a flow rate of "high" using an LSR Fortessa X20 machine, collecting data on BD FACSDiva software. Only runs with cells were evaluated.

DNA damage analysis was conducted on FCS Express 7 software (De Novo Software, Pasadena, CA, USA), and the settings programmed according to Sharma et al. (2020) for sperm cell evaluation. Specifically, a density plot was created to evaluate forward scatter versus side scatter, and a gate was drawn to exclude smaller debris and non-sperm cells. This gate was pulled into another plot to evaluate Phycoerythrin (PE) Texas red staining by forward scatter, where Propidium-iodide (PI) -positive cells were determined (PI stained cells are considered damaged). A histogram was created to count fluorescein isothiocyanate (FITC)-A stained cells. This histogram contained one large peak where the majority of cells fell, and cells to the right tail of this distribution were considered damaged. A quadrant dot plot was also made where the top right quadrant captured the tail of the histogram (indicating percent of damaged cells), and the top left quadrant indicated the percent of undamaged cells.

2.3.7 | Statistical Analysis

2.3.7a | Age

Age was first designated by subtracting the date of birth from the date the sample was collected. Since ferret breeding season is in spring each year and gestation lasts 41-43 days, ferrets are typically born in the summer months (Miller et al., 1996). This means that most ferrets are not yet a year old when they enter the breeding season. However, because they are capable of breeding, they are considered one-year-olds by management. Management uses breeding seasons as a proxy for how old ferrets are and how experienced they are. Therefore, for a more functional age metric to include in models, age was designated by determining how many breeding seasons an individual experienced. The start of the breeding season was designated as February 1st. Therefore, a ferret who is approaching their second birthday but whose sample was taken in March during the breeding season would be considered a two-year-old, since it is his or her second breeding season (even if his or her actual second birthday is several months in the future).

2.3.7b | Vitamin serum analyses

Vitamin E and A results were split into two datasets: one that included "paired" individuals (consisting of individuals that had both "before and "during" diet treatment metrics), and one that included individuals with only one sample each. Both datasets included vitamin E and A results and ferrets across all diets. Vitamin A values were non-normal and so were log-transformed. Linear models in RStudio (RStudio Team, 2020) were used to analyze the paired datasets to determine whether sex, diet, location, age, treatment (before or during diet), or sample year influenced vitamin E and A results. For a predictor in the linear model to be considered for post-hoc tests, a threshold was set at $p=0.10$. Upon post-hoc tests, $p<0.05$ was still the threshold

necessary to be considered significant. Based on significant predictors, datasets were split accordingly and tested using a nonparametric test of location parameters (of the package "nptest" in R), a corollary to the Wilcoxon test for paired samples (before and after experimental treatments) that allows the user to account for heteroskedasticity (Helwig, 2021). A parameter in this test allows the user to assess whether the experimental treatment increases or decreases the median of the result. For the vitamin A location test, we assessed whether median vitamin A levels decreased in the during-treatment period, since studies have shown that vitamin E can antagonize vitamin A levels, and that when vitamin E levels are high, vitamin A may become suppressed (Swick & Baumann, 1952). For the vitamin E location test, we assessed whether median vitamin E levels increased in the during-treatment period, since the vitamin E supplementation was designed to increase circulating vitamin E levels. Medians are better to use in statistical tests for non-parametric data and skewed distributions. There were six control males and eight control females used for paired testing of vitamin A and E analysis (14 total). There were 10 first-gen vitamin E males and females each for paired testing of vitamin A and E analysis (20 total). All p-values reported are for one-sided statistical tests, as we tested whether vitamin levels either significantly increased or decreased, but not both.

Linear models were also analyzed on non-paired datasets to determine significant predictors, and then tested using a Wilcoxon or Kruskal-Wallis tests, followed by a Paired Wilcoxon Test to differentiate significance between group variables. All p-values reported are for one-sided statistical tests. For the individual vitamin A and E analysis, we used the control "before" diet supplementation from the paired dataset and combined that with the single sample dataset from ferrets supplemented with vitamin E to be able to compare diets (since the individual dataset did not have single control individuals available). For the individual vitamin A

and E analysis, there were also no samples from females that were supplemented with vitamin E, so we just included unpaired samples from males supplemented with vitamin E. We therefore did not evaluate sex as a predictor in these male-only model. Thirty-six individuals were included in this analysis.

2.3.7c | TAC serum analysis

TAC serum data organization followed that of the vitamin samples – results were split into a "paired" individual dataset and a dataset that included individuals with only one sample. Two paired analyses were conducted: one for control individuals and one for vitamin E-only individuals (data was not available to evaluate first-generation vitamin E individuals). Sex was included in the linear model for the control paired analysis but removed from the vitamin E analysis since there were no vitamin E females. Further, there were only vitamin E females and no control females for the individual dataset, so females were removed from the individual analysis. Additionally, sample year had to be excluded because it was collinear with diet in the individual analysis (since the generation part of diet correlates with year). Linear models were run on paired and individual datasets to determine whether diet, location, treatment (pre- or during diet), or sample year influenced TAC results. Based on significant predictors, datasets were split accordingly and tested using the Wilcoxon test. For the TAC individual analysis, 33 individuals were included. For the TAC vitamin E individuals paired analysis, 14 individuals were included. For the TAC control individual paired analysis, 22 individuals were included.

To evaluate whether TAC serum levels influence fertility status, reproductive records were joined with the TAC serum records and TAC serum was used as a predictor for whether an individual successfully paired (female whelped) that sample year (n=40).

2.3.7d | TAC seminal fluid analysis

TAC seminal fluid data only contained two individuals that had repeat measurements, so this dataset was treated as the "individual" datasets in the previous analyses. This dataset also included samples from four wild individuals. A linear model was used to analyze the dataset to determine whether sex, diet, location, treatment (before or during diet), or sample year influenced TAC seminal fluid results. A Kruskal-Wallis test and then pairwise Wilcoxon tests were used to determine significant differences between the dependent variables. 55 individuals were included in this analysis.

To evaluate whether TAC seminal fluid levels influence fertility status, reproductive records were joined with the TAC seminal fluid records and TAC serum was used as a predictor for whether an individual successfully paired (female whelped) that sample year (n=35).

2.3.7e | Seminal Traits Analysis and Correlation Tests

Vitamin E, Vitamin A, TAC serum and TAC seminal fluid results were each matched with seminal traits using ID and date where data were available (n=19). Generalized linear mixed models were constructed for percent motility, percent normal morphology, motility status, percent abnormal acrosome, total testes volume, and sperm concentration (mill/mL), with serum vitamin A content, serum vitamin E content, and diet as predictors and sample year as a random effect.

Kendall correlation tests were used to determine whether there existed an association between vitamin and TAC levels and sperm metrics, including percent motility, percent normal morphology, motility status, percent abnormal acrosome, total testes volume and sperm concentration.

To determine whether increased plasma TAC levels implied increased seminal fluid TAC levels, Kendall correlation tests were run between TAC serum and TAC seminal fluid. These two separate datasets had to be joined; 14 records were matched.

2.3.7f | Sperm DNA damage

Results regarding percent of damaged cells were exported into Excel and evaluated in R Studio. We hypothesized that sperm from individuals with above-average levels of DNA damage would result in a decline in fertility. To test this, we compared the ratio of damaged to undamaged cells in each sample to the third quartile levels of damaged and undamaged sperm cells in our study using a one-sided Fisher's Exact Test, allowing us to categorize cells as "significantly damaged" or not relative to the median. This allowed us to test if DNA damage above a critical threshold, regardless of absolute percentage, would affect fertility metrics of interest, by using this result as a categorical variable in our linear model, rather than as a continuous trait. The ratio of damaged to undamaged cells was not a predictor of the sperm metrics; therefore, we used log percent DNA damage as a predictor to determine its effects on sperm metrics.

Percent DNA damage was not normally distributed, so was log transformed. A linear model was then constructed to determine what factors may predict log percent DNA damage. The GLMM with the lowest AIC score was constructed with diet, total number of cells, and sperm per microliter as predictors for log percent DNA damage. Total number of cells and sperm concentration were included to determine whether the more cells present in a sample influenced the percent DNA damage that was detected. 64 individuals were evaluated.

Log percent DNA damage records were also combined with data on reproductive outcomes for pairings that males had, where available (n=40). Percent whelped was calculated for all available reproductive records by dividing number of whelped outcomes over total outcomes. Number of kits born was also determined from these records. Both percent whelped and number of kits born were not normally distributed but not transformed so as to preserve the zero values. These were tested in a linear model to determine whether percent DNA damage and other factors influenced them. Post-hoc tests including Kruskal–Wallis and Wilcoxon tests were run after significant predictors were determined using linear models.

Kendall correlation tests were run to determine whether percent DNA damage was correlated with sperm parameters (n=65). Additionally, a linear model was built to determine whether sperm parameters including DNA damage influenced total kits born and percent whelped (n=42). Lastly, sperm parameters were included in linear models to determine whether and how they changed with diet treatments (n=65). A full model with diet, sperm parameters and percent DNA damage could not be built to predict percent whelped and number of kits born because the dataset became increasingly small and the number of predictors increased, which causes overfitting and reduces model accuracy. For this reason, we used two separate models for predicting percent whelped and number of kits (one model using diet information, the other model focusing on sperm parameters).

To determine whether serum vitamin levels or TAC in serum or seminal fluid influences sperm DNA damage, linear models were built with DNA damage as the dependent variable and breeding year, diet and TAC serum or seminal fluid as predictors. An interaction term between TAC serum or seminal fluid and diet was included in order to parse out the relationship they both had on DNA damage. There were few records that matched between the DNA damage and

serum and seminal fluid datasets. For example, models that predicted whether DNA damage was influenced by serum vitamin and TAC levels had very few records (vitamin A/E: n=15; serum TAC: n=19). Matching with seminal fluid TAC had more records (n=49). Kendall correlation tests were run between log percent DNA damage and serum vitamin levels, TAC serum and TAC seminal fluid for further investigation into relationships.

2.4 | Results

2.4.1 | Vitamin A Serum – Paired individuals

The linear model determined that sex influenced ($p < 0.005$) vitamin A levels in the control treatment group (Table 2.1). Females had higher vitamin A levels than males both before ($W = 48$, $p < 0.005$) and during ($W = 48$, $p < 0.005$) treatments (Fig. 2.1). Therefore, control diet males and females were analyzed separately. We determined that vitamin A levels did not decrease in the during-treatment period compared to the pre-treatment period for either males ($t = -1.15$, $p = 0.89$) or females ($t = 0.98$, $p = 0.14$).

Table 2.1. Model parameters from the fitted linear model testing for the influence of sex on serum vitamin A in paired control diet ferrets

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	2.78	0.13	21.98	**<0.00001**
Sex: Male	-1.20	0.19	-6.21	**<0.00001**

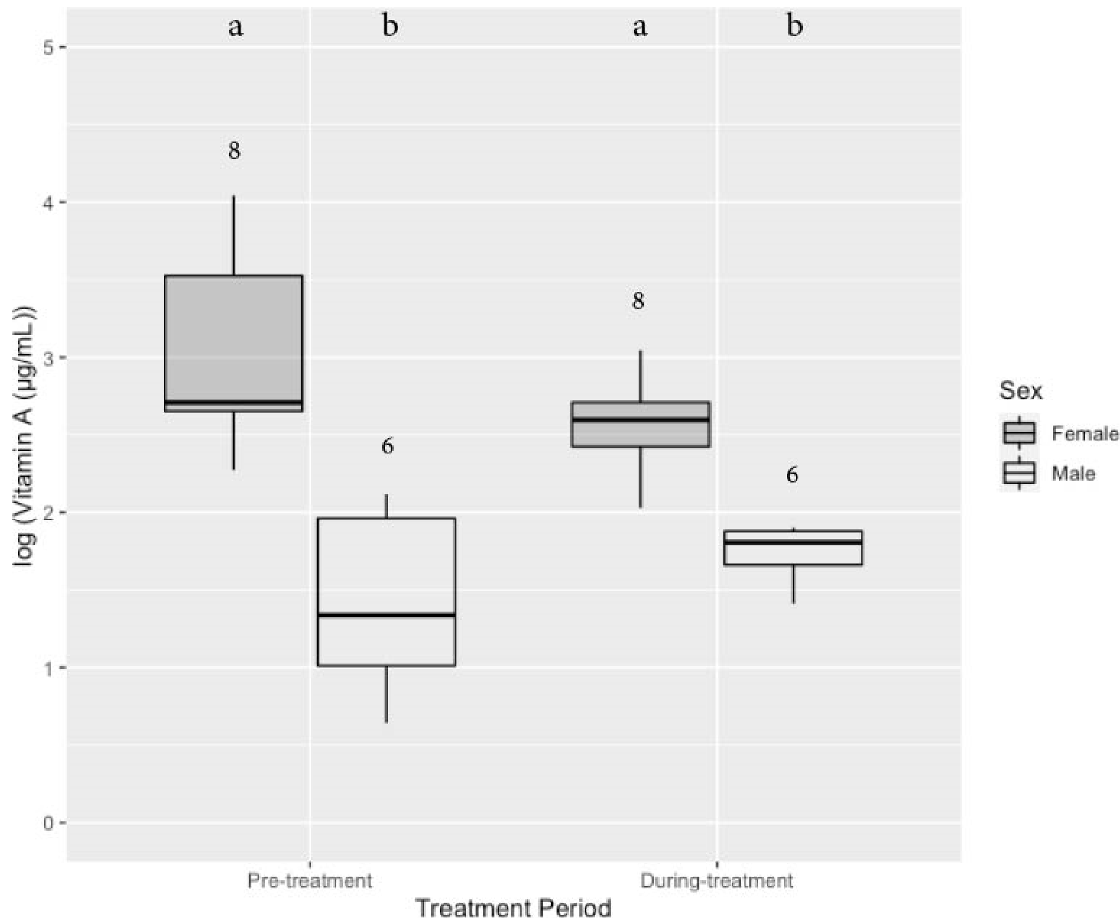

Figure 2.1. Serum vitamin A levels between in control treatment males and females for paired analysis. Number above bars is the number of male or female ferrets in that treatment. Superscripts indicate differences ($p<0.05$) between sexes and treatment periods. Boxes represent the middle 50% of data; edges are the 25th and 75th percentiles. Dark lines in middle of the box represent the median.

Sex also influenced ($p<0.005$) serum vitamin A levels in the first-gen vitamin E diet ferrets (Table 2.2). Specifically, females had higher vitamin A levels than males in both the before ($W = 85$, $p < 0.01$) and during-treatment ($W = 97$, $p < 0.001$; Fig. 2.2). Therefore, we analyzed males and females separately and found that serum vitamin A levels decreased from the pre- to during-treatment in both males ($t = 2.1$, $p < 0.05$) and females ($t = 1.78$, $p < 0.05$; Fig. 2). There were only four males to evaluate on the carcass diet; vitamin A levels did not change across the sampling period ($t = 0.73$, $p = 0.31$) (mean, 1.22 ±0.31; range, -0.12-2.83 µg/mL).

Table 2.2. Model parameters from the fitted linear model testing for the influence of sex on vitamin A in paired first-gen vitamin E diet ferrets

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	2.80	0.15	18.76	**<0.00001**
Sex: Male	-1.142	0.21	-5.41	**<0.00001**

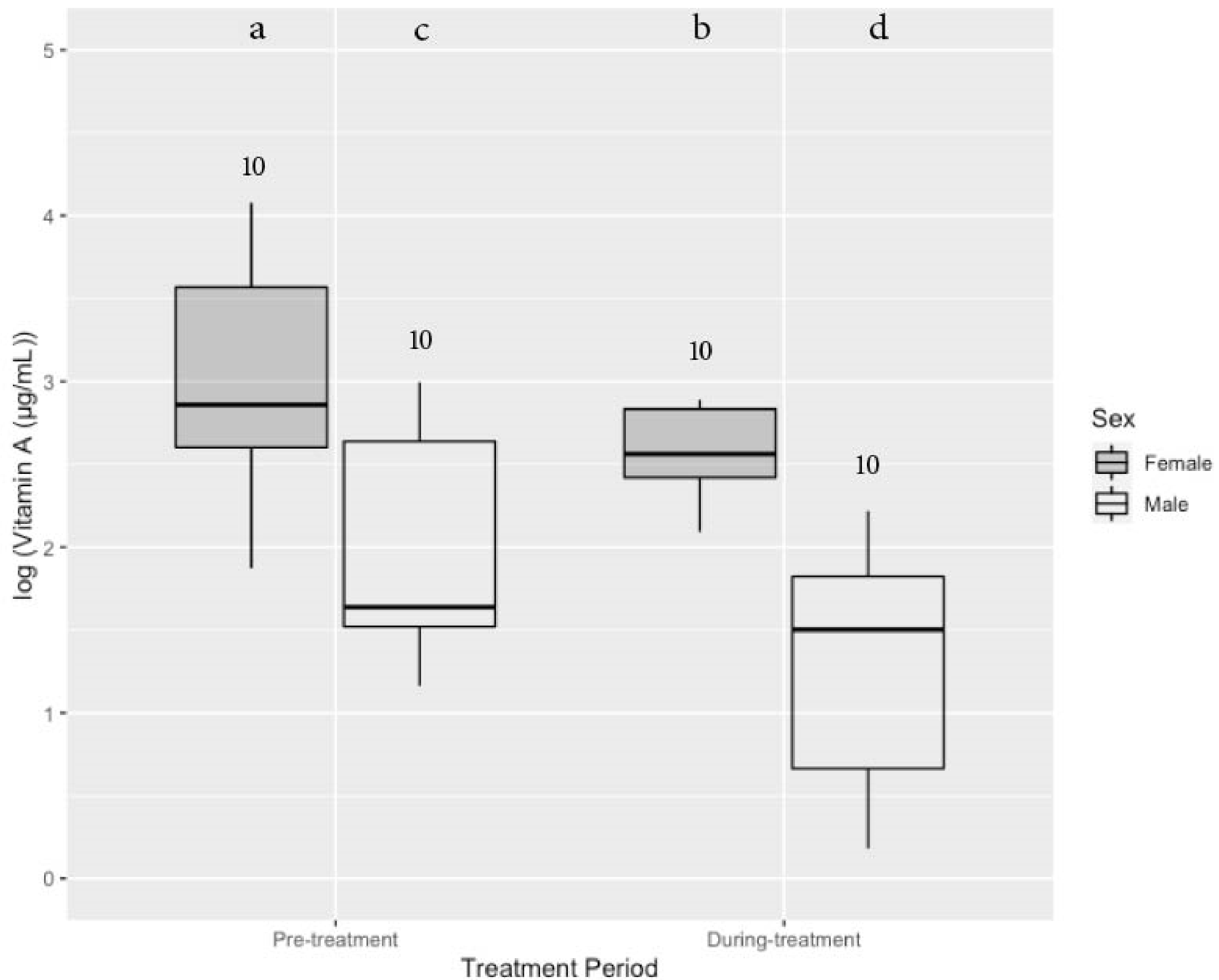

Figure 2.2. Serum vitamin A levels between sexes and pre- and during-supplementation of vitamin E in the diet for first-gen vitamin E paired analysis. Number above bars is the number of male or female ferrets in that treatment. Superscripts indicate differences ($p<0.05$) between sexes and periods of supplementation of vitamin E.

2.4.2 | Vitamin A Serum – Individual analysis

The linear model determined that age ($p<0.005$) was a predictor of vitamin A serum levels, and year ($P<0.05$) passed the threshold for further investigation via post-hoc tests (Table 2.3). Further analysis determined that serum across ages varied ($\chi^2_5 = 19.62$, $p < 0.005$). Specifically, one-year old males had higher serum vitamin A levels than three-year old males ($p<0.05$), but were similar ($p<0.05$) across other ages (Fig. 2.3). Serum vitamin A levels also varied ($\chi^2_3= 11.88$, $p<0.05$) across the years. Specifically, serum vitamin A levels from 2017 were higher ($p<0.05$) than 2021, but both were similar ($p<0.05$) to 2018 and 2019 (Fig. 2.4).

Table 2.3. Model parameters from the fitted linear model testing for the influence of age, diet, sample year and location on serum log vitamin A levels in control, first- and second-gen vitamin E ferrets

Fixed effects	**Estimate**	**Standard Error**	**t-value**	**p-value**
(Intercept)	2.33	0.203	11.47	**<0.0001**
First Gen. Vit. E	-0.04	0.75	-0.05	0.95934
Second Gen. Vit E	1.91	0.61	0.32	0.75025
Age	-0.47	0.12	-3.90	**0.00056**
Sample Year 2018	2.53	0.93	2.72	**0.01106**
Sample Year 2019	-0.60	0.65	-0.92	0.36372
Sample Year 2021	-0.67	0.51	-1.32	0.19922
Location: LZG	-0.05	0.33	-0.15	0.88079

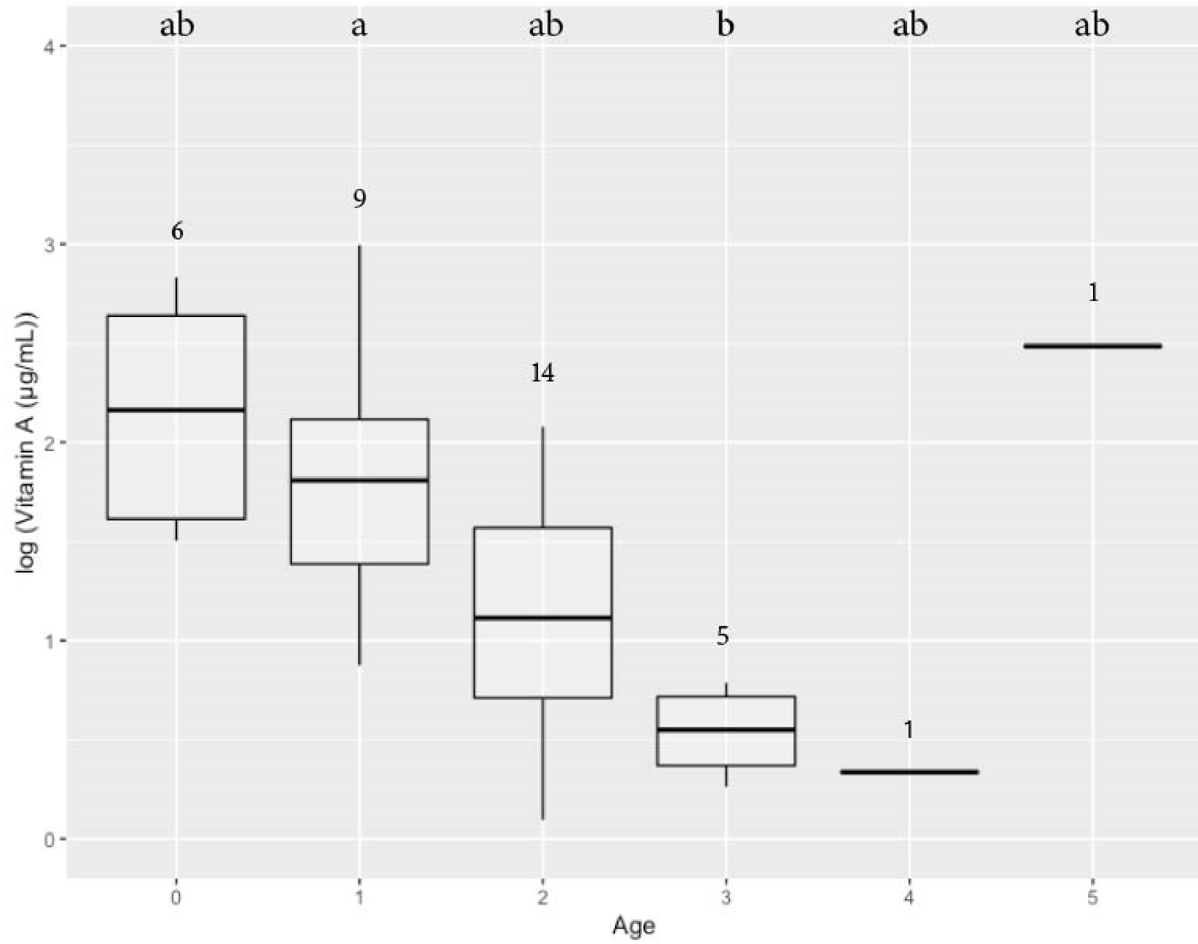

Figure 2.3. Ferret serum log vitamin A levels across age for all diet treatments combined in individual analysis. Number above bars is the number of ferrets in that treatment. Superscripts indicate differences ($p<0.05$) in serum vitamin A levels across the age of ferrets in the diet study.

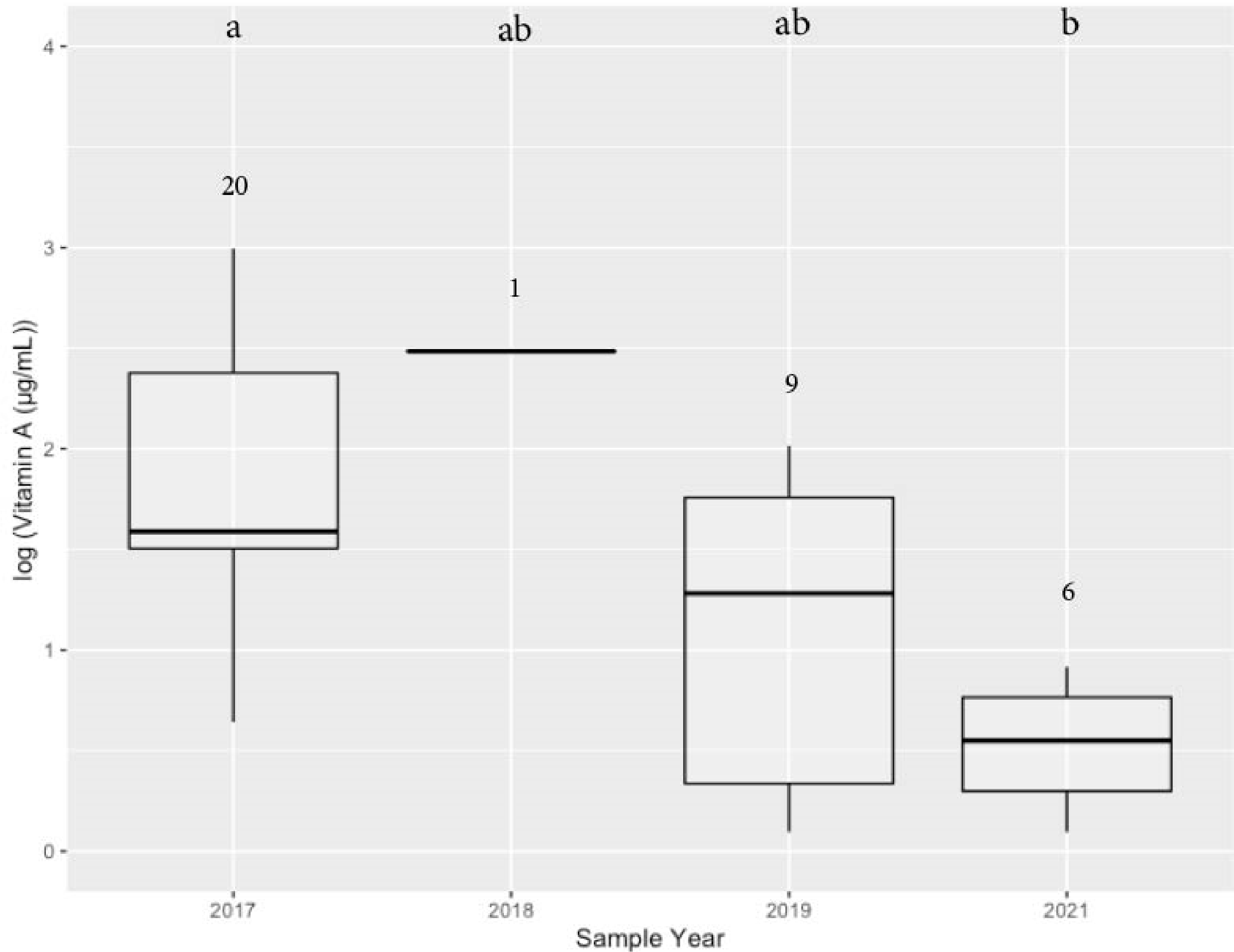

Figure 2.4. Ferret serum vitamin A levels across year for all diet treatments combined in individual analysis. Number above bars is the number of ferrets in that treatment. Superscripts indicate differences ($p<0.05$) in serum vitamin A levels across the years of the diet study.

2.4.3 | Vitamin E Serum – Paired individuals

Sex did not influence vitamin E levels in either the control diet ($p= 0.17$) or first-gen vitamin E diet ferrets ($p= 0.14$); therefore, we combined male and female data for both the control individual analysis and the first-gen vitamin E individuals analysis. For the control treatment, vitamin E levels did not increase ($t = -0.35$, $p = 0.391$) during the treatment period compared to before supplementation (mean, 14.5 ± 0.85 µg/mL; range, 9.3- 27 µg/mL). For the first-gen vitamin E diet ferrets, vitamin E serum levels increased ($t = -3.21$, $p< 0.001$) during the

treatment diet period compared to pre-supplementation (Fig. 2.5). However, vitamin E serum levels did not increase (t = 1.46, p = 0.94) from the control to carcass diet period (mean, 11.5 ± 1.90; range, 3.4 - 20 μg/mL).

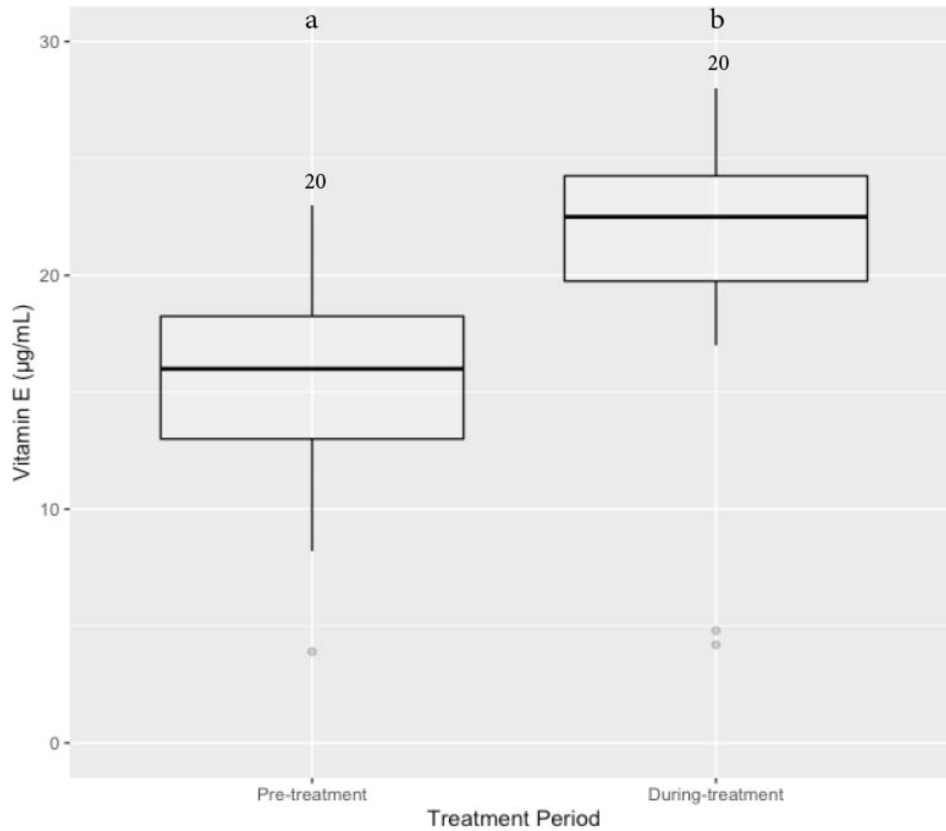

Figure 2.5. Serum vitamin E levels for first-gen vitamin E ferrets before and during vitamin E supplementation. Number above bars is the number of male and female ferrets in that treatment. Superscripts indicate differences (p<0.05) periods of supplementation of vitamin E.

2.4.4 | Vitamin E Serum – Individual analysis

The linear model found that none of the factors (diet, sample year, age or location) affected serum vitamin E levels. Therefore, no further analyses were conducted.

2.4.5 | TAC Serum– Paired individuals

For the control treatment, sex was not a predictor ($p=0.608$) for TAC level; therefore, control males and females were combined. TAC levels of control ferrets were nearly significantly different ($t = -1.69$, $p = 0.051$) before and during-treatment diet (mean, 5073 ± 166 nmol/well; range, 3963 - 6653nmol/well). For both first- and second-generation vitamin E only males, TAC levels were similar ($p=0.083$); therefore, we combined their results. For these vitamin E-supplemented ferrets, TAC did not increase ($t = -1.48$, $p = 0.094$) when comparing the pre-diet (which included first-gen vitamin E only) and during-diet samples (mean, 5118 ± 176 nmol/well; range, 4345 - 6520 nmol/well).

2.4.6 | TAC Serum– Individual analysis

The linear model determined that diet ($p<0.0001$) and age ($p<0.010$) influenced serum TAC levels (Table 2.4). Specifically, second-gen vitamin E individuals had lower ($\chi^2_2 = 11.41$, $p<0.005$) TAC levels than first-gen vitamin E ($p<0.005$) and control ferrets ($p<0.05$) (Fig. 2.6). Age was not found to be a significant factor upon post-hoc testing ($\chi^2_6 = 9.97$, $p<0.10$).

Table 2.4. Model parameters from the fitted linear model testing for the influence of age and diet on serum TAC levels in control, first- and second-gen vitamin E ferrets

Fixed effects	**Estimate**	**Standard Error**	**t-value**	**p-value**
(Intercept)	5228.83	394.14	13.27	**<0.0001**
Age	174.59	100.15	1.74	**0.089**
First Gen. Vit E	-50.42	334.73	-0.15	0.881
Second Gen. Vit E	-1809.85	383.45	-4.72	**<0.0001**
Sex: Male	219.56	361.77	0.61	0.55

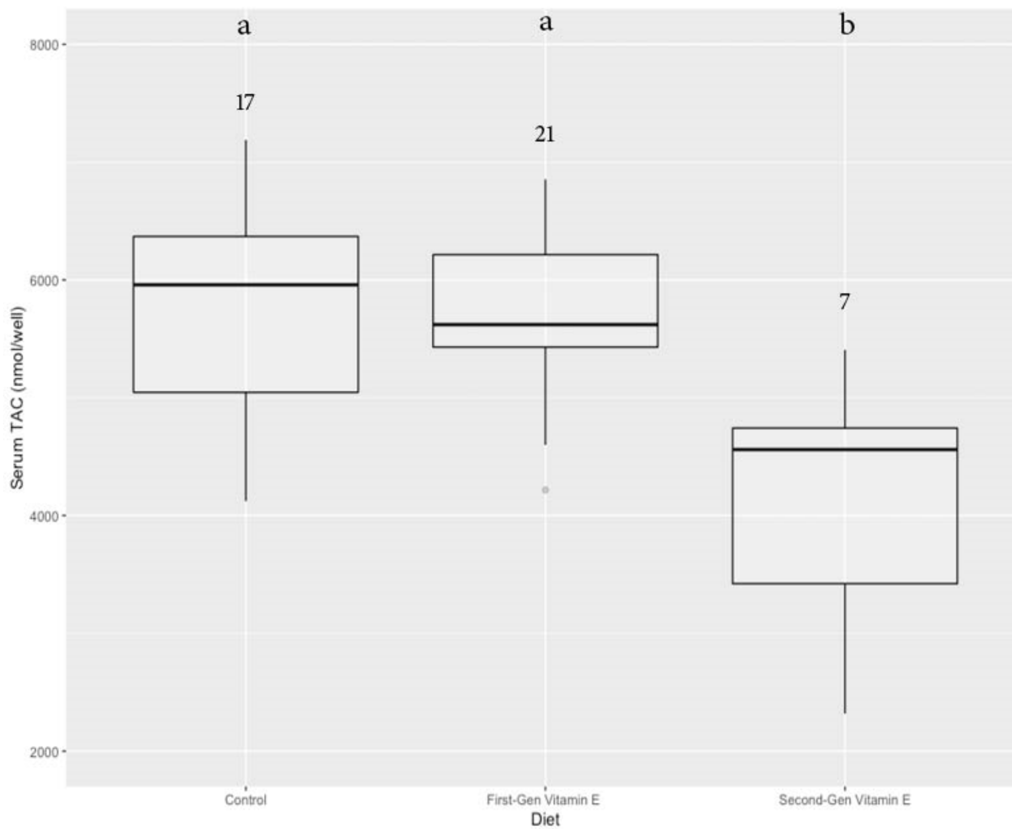

Figure 2.6. Serum Total Antioxidative Capacity (TAC) levels across diet treatments in individual ferret analysis. Number above bars is the number of ferrets in that treatment. Superscripts indicate differences ($p<0.05$) in serum vitamin A levels across diet treatments.

2.4.7 | TAC Seminal plasma – Individual analysis

The model results determined that location was a predictor ($p< 0.01$) of TAC seminal plasma levels (Table 2.5). However, LZG TAC seminal plasma levels (mean, 18.5 ± 2.46; range, 14.3 - 27.3 nmol/well) was nearly higher ($\chi^2_2 = 5.71$, $p = 0.058$) than the other locations (mean, 13.8 ± 0.78 nmol/well; range, 4.13 - 29.3 nmol/well), which were similar ($p>0.60$) to each other. Within individuals, TAC seminal plasma and TAC serum were not correlated ($z = 0.77$, $p = 0.44$).

Table 2.5. Model parameters from the fitted linear model testing for the influence of age, diet, location and sample year on seminal fluid TAC levels in control, first- and second-gen vitamin E ferrets

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	8.14	3.78	2.15	**0.0369**
Location: LZG	7.24	2.67	2.72	**0.0092**
Control diet	1.16	3.28	0.36	0.7254
First Gen. Vitamin E	4.07	3.62	1.12	0.2679
Second Gen. Vitamin E	3.72	3.48	1.07	0.2911
Age	0.21	0.77	0.28	0.7825
Sample year: 2021	2.84	1.79	1.59	0.1194

2.4.8 | TAC fertility status analyses

Neither TAC serum nor TAC seminal plasma were predictive of fertility status (TAC serum (fertile mean, 5486 ± 212 nmol/well, 4217 - 7188 nmol/well; infertile mean, 5315 ± 238 nmol/well, 2403 - 7047 nmol/well); TAC seminal plasma (fertile mean, 12.0 ± 1.06 nmol/well, 4.13 - 25.3 nmol/well; infertile mean, 14.6 ± 1.30, range, 8.90 - 23.8 nmol/well)).

2.4.9 | Sperm parameters, vitamin serum and TAC

According to the GLMM, sperm metrics nor serum and seminal plasma TAC data were significantly influenced by vitamin serum. Additionally, there were no significant correlations found between serum vitamin A, serum vitamin E and serum and seminal fluid TAC results and all sperm metrics (n=57): ((motility: mean, 56.23 ± 1.48%; range, 0-70%); sperm normality: mean, 32.14 ± 2.44%; range, 2-73%); sperm forward progression: mean, 2.90 ± 0.04; range, 1-3.5); abnormal sperm acrosome: mean, 21.02 ± 2.11%; range, 0-62%); testes volume: mean, 1.34 ± 0.028 cm^3; range, 0.59-1.73 cm^3); sperm concentration: 332.58 ± 26.17x10^6 sperm/mL; range, 13.1 - 919.4 x10^6/mL).

2.4.10 | Sperm DNA Damage

The linear model determined that control ($p<0.05$) and first-generation vitamin E diets were predictors ($p<0.001$) of DNA damage (Table 2.6). Specifically, second-generation vitamin E ferrets had lower ($\chi^2_4 = 17.29$, $p< 0.005$) percent DNA damage than first-generation vitamin E ferrets (Fig. 2.7). Total events passed the threshold for post-hoc testing ($p=0.05$); however, the number of reproductive events was uncorrelated with DNA damage ($z = 0.85$, $p = 0.39$).

Table 2.6. Model parameters from the fitted linear model testing for the influence of diet, total events, and sperm per ul on percent sperm DNA damage.

Fixed effects	**Estimate**	**Standard Error**	**t-value**	**p-value**
(Intercept)	0.01	0.05	0.27	0.79005
Total events	0.00	0.00	2.00	**0.05043**
Sperm per ul	<0.00	0.00	-1.13	0.26470
Control diet	1.11	0.05	2.24	**0.02878**
First Gen. Vitamin E	1.95	0.52	3.75	**0.00042**
Second Gen. Vitamin E	0.31	0.53	0.58	0.56632
Wild diet	0.62	0.71	0.87	0.38809

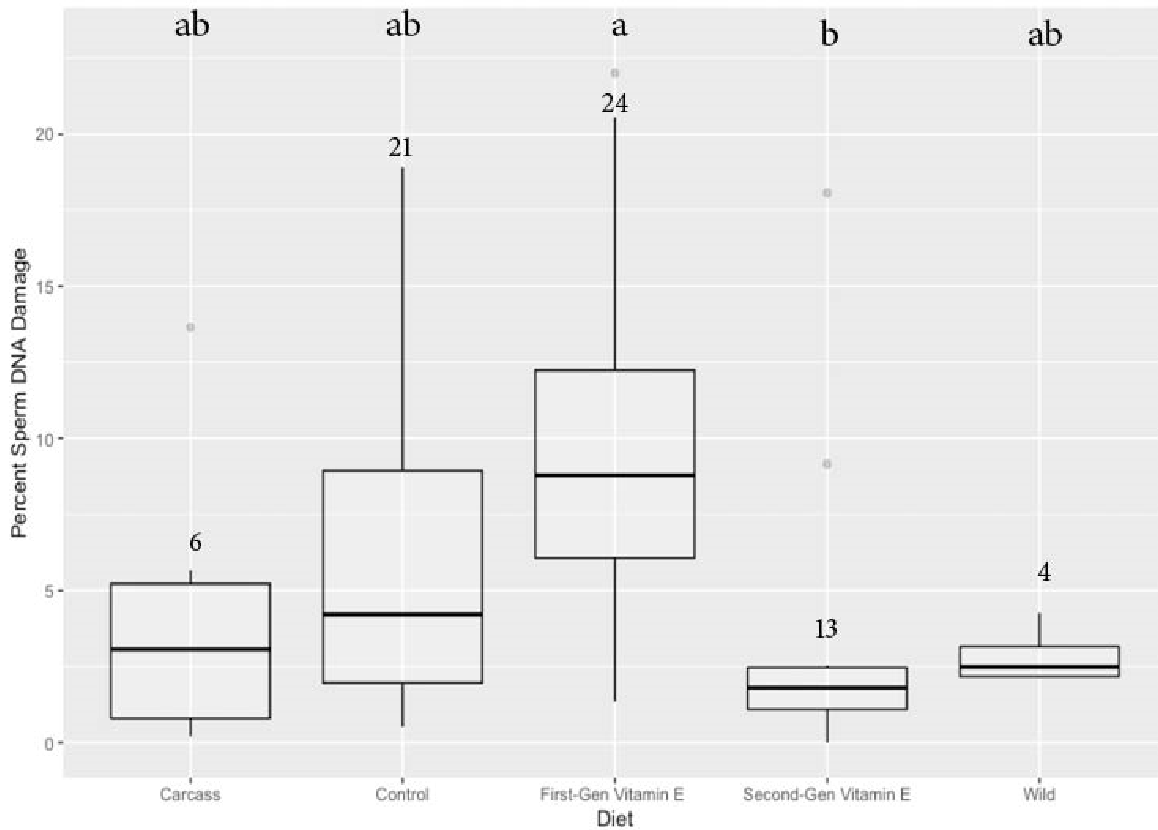

Figure 2.7. Percent sperm DNA damage across diet treatments (including wild individuals) in individual analysis. Number above bars is the number of ferrets in that treatment. Superscripts indicate differences ($p<0.05$) sperm DNA damage (%) across diet treatments.

Identical models as described above were used to determine predictors of percent whelped and number of kits born per male. There were no significant factors in these models (mean percent whelped: 0.42± 0.070; range, 0-1; mean kits born: 3.68± 0.74; range, 0-21).

Sperm DNA damage was negatively correlated with percent motility ($z = -2.12$, $p<0.05$), percent normal sperm ($z = -2.96$, $p<0.005$) and percent normal acrosome ($z = -2.72$, $p<0.05$), and positively correlated with percent tail coil ($z = 2.68$, $p<0.05$) and percent damaged apical ridge ($z = 2.94$, $p< 0.005$). There were no significant correlations between percent DNA damage and motility status, percent bent midpiece with droplet, percent bent tail with droplet, percent bent tail without droplet, percent proximal droplet, percent distal droplet, or total testes volume. When

investigating whether the percent DNA damage was influenced by serum vitamin A and E levels, breeding year was a predictor ($p<0.05$) in the linear model but serum vitamin A and E were not (Table 2.7). Upon further investigation, 2021 levels (mean, 0.74 ± 0.24; range, 0.13 - 1.62) were lower ($p<0.005$) than 2019 (mean, 2.20 ± 0.25; range, 0.70 - 3.23). Neither serum vitamin A nor E were correlated with percent DNA damage.

Table 2.7. Model parameters from the fitted linear model testing for the influence of serum vitamin levels, breeding year and diet on percent sperm DNA damage

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	2301.63	880.78	2.61	**0.0310**
Serum Vitamin E	0.02	0.03	0.51	0.6230
log(Serum Vitamin A)	-0.21	0.67	-0.32	0.7600
Breeding Year	-1.14	0.44	-2.61	**0.0310**
Diet: Carcass	-0.64	1.13	-0.56	0.5900
Diet: First gen vitamin E	-1.61	1.12	-1.43	0.1900
Diet: Second gen vitamin E	-0.75	0.65	-1.14	0.2870

In the linear model, breeding year predicted ($p<0.001$) whether percent DNA damage was influenced by serum TAC levels or an interaction between TAC serum and diet (Table 2.8). Specifically, DNA damage levels in 2018 were higher than both 2019 ($p<0.05$) and 2021 ($p<0.005$), and 2019 was also higher than 2021 ($p<0.05$; Fig. 2.8). Serum TAC was not significant in the model. However, DNA damage and serum TAC correlated positively ($r=0.47$, $p<0.05$; Fig. 2.9).

Table 2.8. Model parameters from the fitted linear model testing for the influence of TAC serum levels, breeding year and diet on percent sperm DNA damage

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	1600	296.7	5.39	**0.00016**
TAC serum level	0.00	0.00	0.55	0.59068
Diet: First gen vitamin E	1.42	1.87	0.76	0.46263
Diet: Second gen vitamin E	1.79	1.88	0.95	0.35921
Breeding year	-0.79	0.15	-5.40	**0.00016**
TAC* First gen vitamin E	<0.00	0.00	-0.74	0.47140
TAC* Second gen vitamin E	<0.00	0.00	-0.96	0.35811

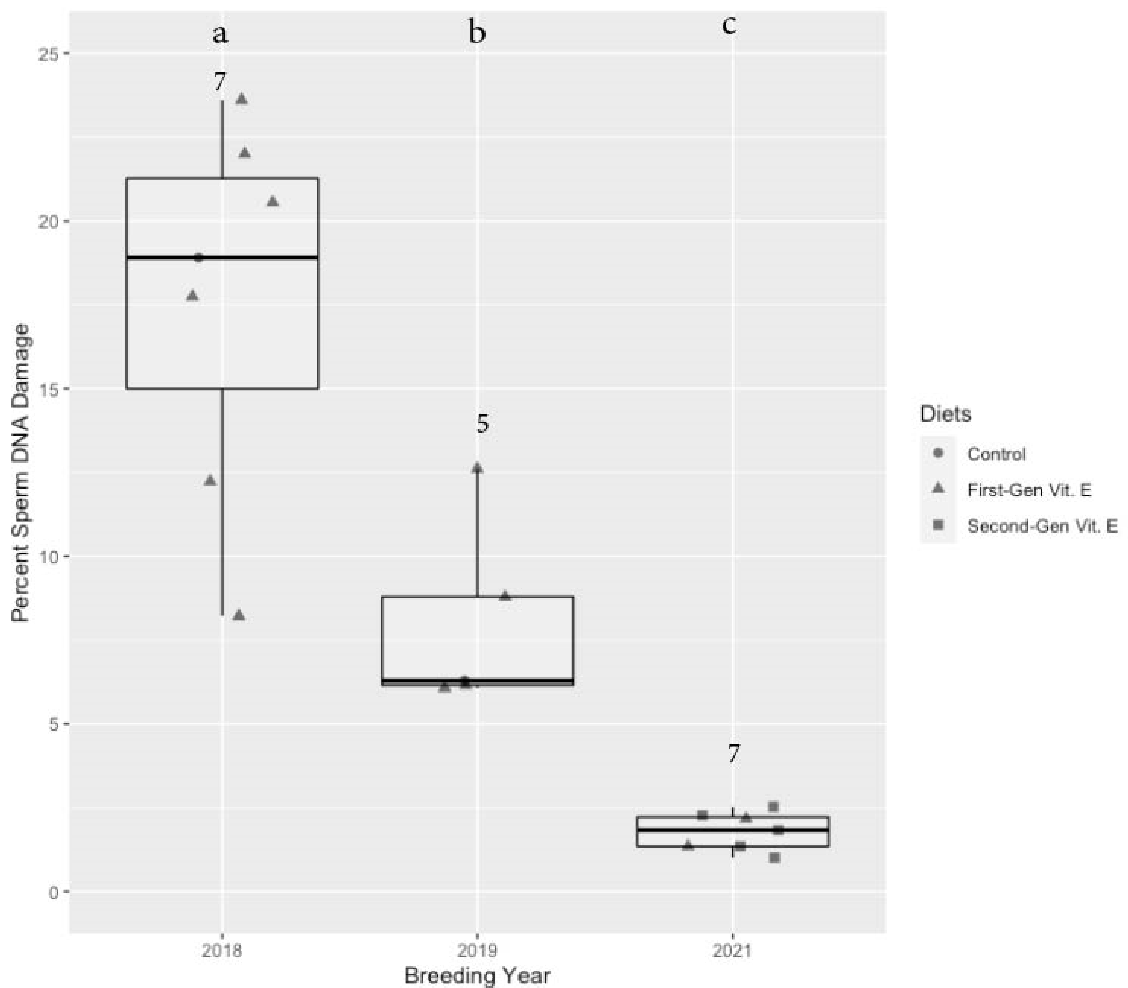

Figure 2.8. Percent DNA damage across breeding years with diet treatment as different shaped points. Number above bars is the number of ferrets in that diet. Superscripts indicate differences ($p<0.05$) in sperm DNA damage (%) across breeding year.

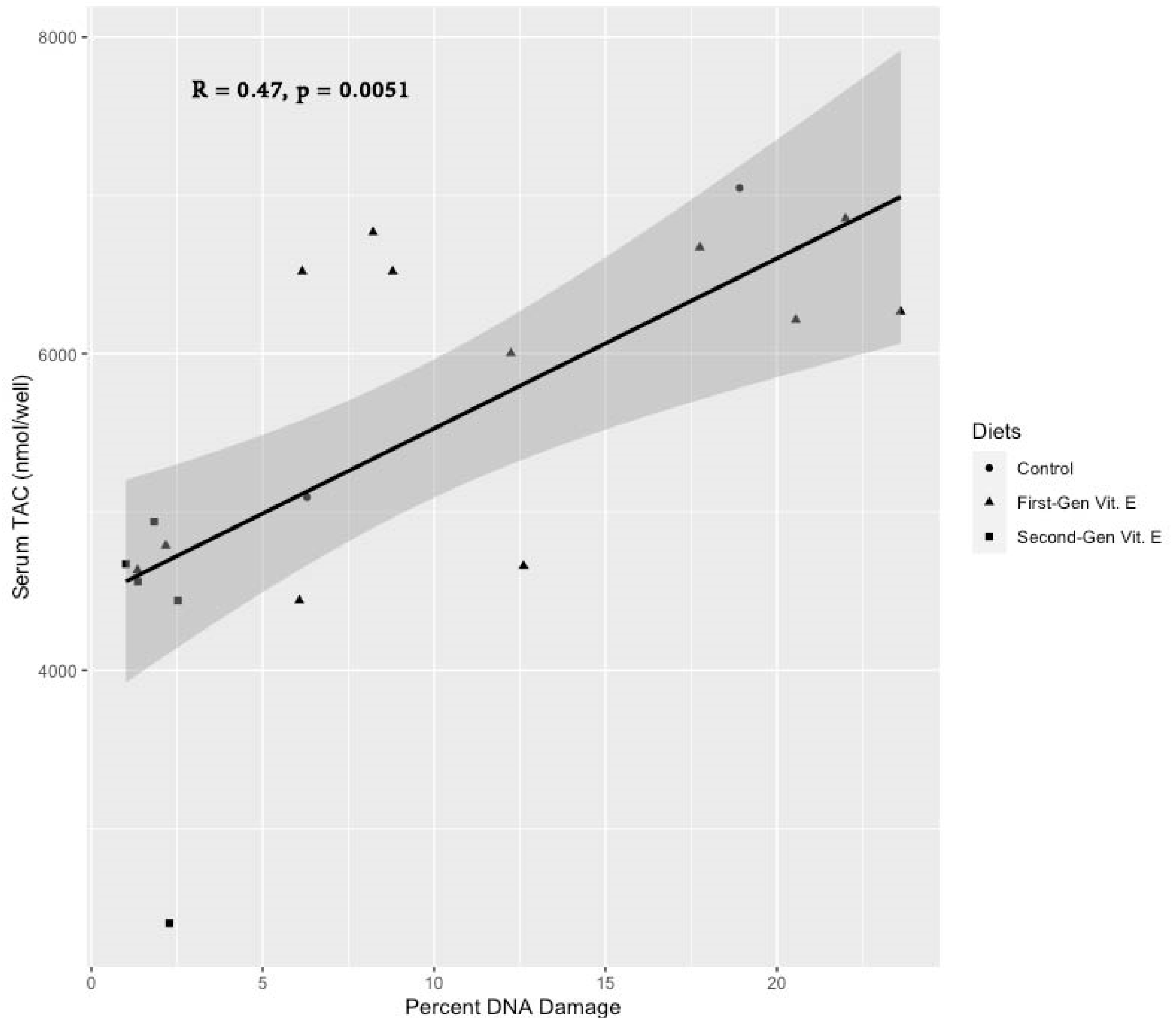

Figure 2.9. Correlation of Percent DNA damage and serum TAC. Diet treatments are represented by shaped dots. R and p-value provided.

Percent DNA damage was influenced by carcass diet ($p<0.05$), the interaction between TAC seminal plasma and the carcass diet ($p<0.05$), and breeding year ($p<0.10$) (Table 2.9). Post-hoc testing looking at difference across diets for DNA damage ($\chi^2_3 = 9.65$, $p<0.05$) also found that second-gen vitamin E was lower than first-gen. Percent DNA damage and seminal plasma TAC values were negatively ($r=-0.26$, $p<0.05$) correlated (Fig. 2.10). Breeding year influenced

DNA damage (χ^2_1 = 19.05, p<0.0001); specifically, 2021 had lower DNA damage levels than 2019 (p<0.0001).

Table 2.9. Model parameters from the fitted linear model testing for the influence of TAC seminal fluid levels, breeding year and diet on percent sperm DNA damage

Fixed effects	**Estimate**	**Standard Error**	**t-value**	**p-value**
(Intercept)	538.47	317.06	1.70	**0.09742**
TAC seminal fluid level	-0.05	0.04	-1.31	0.19736
Diet: Carcass	-4.54	1.37	-3.31	**0.00200**
Diet: First gen vitamin E	0.29	0.75	0.39	0.69900
Diet: Second gen vitamin E	-0.31	0.85	-0.37	0.71556
Breeding Year	-0.27	0.16	-1.69	**0.09880**
TAC*Carcass	0.30	0.10	3.00	**0.00464**
TAC* First gen vitamin E	<0.00	0.05	-0.12	0.90408
TAC* Second gen vitamin E	<0.00	0.06	-0.34	0.73672

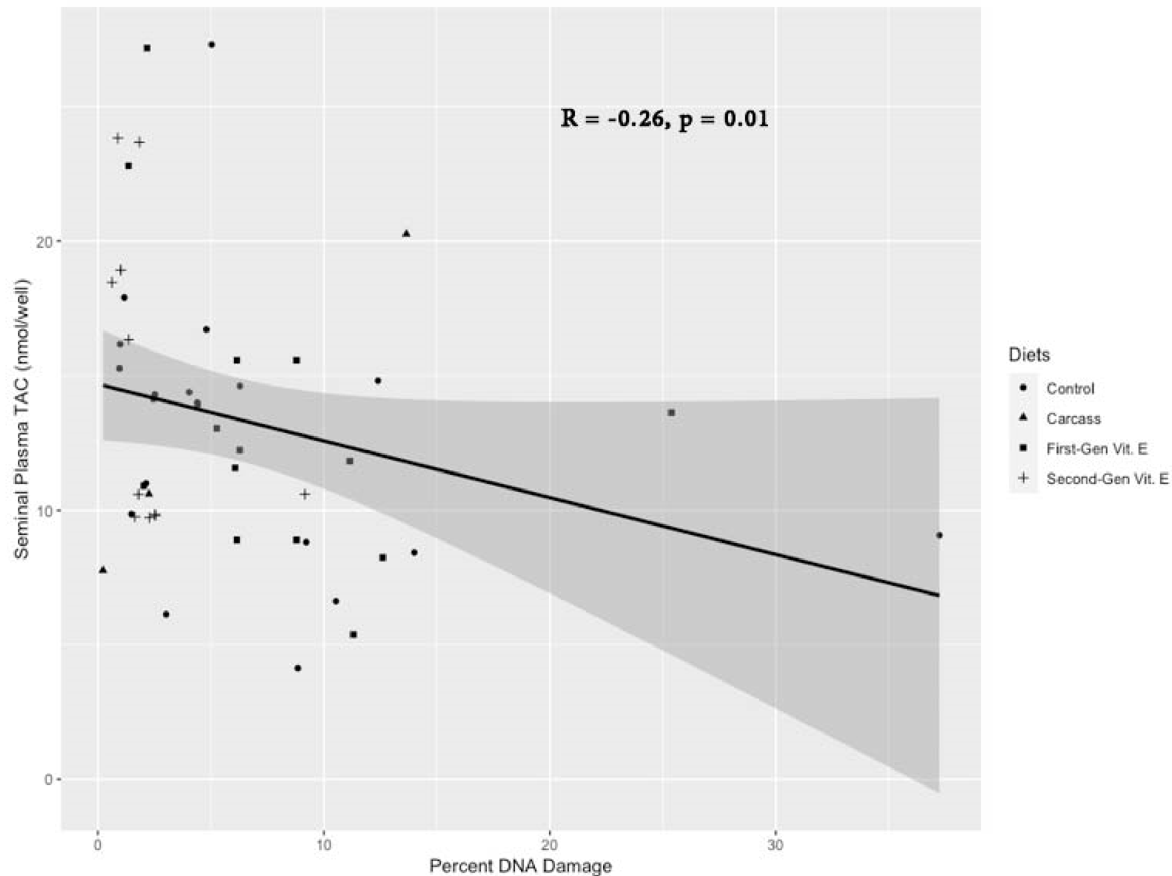

Figure 2.10. Correlation of percent DNA damage and seminal plasma TAC. R and p-value provided.

2.4.11 | Sperm parameters, diet and breeding year

In the linear model, predictors such as breeding year and diet were initially found to be initially significant and predictive (Table 2.10) of the following sperm traits but were not significant ($p<0.05$) upon post-hoc testing: sperm motility ($\chi^2_4 = 5.47$, p = 0.24; Table 2.10a), percent of sperm with bent midpieces ($\chi^2_3= 7.60$, p = 0.06; Table 2.10e; further pairwise Wilcoxon testing found no significant difference between breeding years), progressive sperm motility ($\chi^2_4 = 5.14$, p = 0.27; Table 2.10b), bent midpiece without droplet ($\chi^2_4 = 4.08$, p = 0.40; Table 2.10f), proximal droplet ($\chi^2_4 = 7.67$, p = 0.10; Table 2.10h), and percent of distal droplets

(χ^2_4 = 10.07, p = 0.04; Table 2.10i; further pairwise Wilcoxon testing found no significant difference between diets). There was no significant influence of diet or breeding year in the linear model on tail coil, bent tail with droplet or total testes volume.

Percent normal sperm, percent abnormal acrosome, bent tail without droplet and DAR differed significantly with died after post-hoc tests. Because wild ferrets were the only samples taken in 2020, breeding year confounded some of the findings. Overall, percent normal sperm was significantly influenced by breeding year (χ^2_3= 16.96, p < 0.001; Table 2.10c & 2.11a) and the wild diet (χ^2_4= 10.03, p = 0.04; Tables 2.10c & 2.11b). However, upon further post-hoc testing, percent normal sperm between the control and wild diet (p=0.07) and first-gen vitamin E diet and wild diet (p= 0.07) were not found to be significant.

The percent abnormal acrosome was significantly influenced by the wild diet and breeding year (Table 2.10d). Specifically, it varied (χ^2_4= 11.93, p = 0.02) among the wild and control diets with wild ferrets having a lower ($p<0.05$) percentage of abnormal acrosome than control ferrets (Table 2.11b). Breeding year varied (χ^2_3= 15.34, $p<0.05$) where 2020 saw lower levels of abnormal acrosome than 2018, 2019 and 2021 ($p<0.05$) (only wild ferrets were sampled in 2020). The year 2021 was also significantly lower than 2018 ($p<0.05$).

The linear model also found that the percentage of sperm with bent tails without droplet were significantly influenced by the carcass and first-gen vitamin E diets as well as breeding year (Table 2.10g). Bent tails without droplet were more prevalent (χ^2_4= 12.97, p = 0.01) in the control diet compared to the first-gen vitamin E ferrets ($p<0.05$). Breeding year was not significant upon post-hoc testing (χ^2_3= 1.99, $p<0.05$). The percentage of sperm with damaged apical ridges was significantly influenced by the wild diet in the linear model (Table 2.10j) and

upon post-hoc testing (χ^2_4 = 11.53, p = 0.021) wild had a lower (p<0.05) percentage than control ferrets (Table 2.11b).

None of the following sperm metrics affected (p>0.05) the number of kits born and percent females who whelped: percent DNA damage (mean, 7.56 ± 1.21; range, 0-37.2), percent motility (mean, 55.2 ± 1.95; range, 0-70), percent normal morphology (mean, 27.4 ± 2.81; range, 2-60), percent motility status (mean, 2.84 ± 0.0915; range, 0-3.5), abnormal acrosome (mean, 25.2 ± 2.82; range, 0-70), pre-freeze damaged apical ridge (mean, 23.7± 2.79; range, 0-65), tail coil (mean, 0.68 ± 0.16; range, 0-4) and total testes volume (mean, 1.35 ± 0.03; range, 1.1 - 1.68).

Table 2.10: Model parameters from the fitted linear model for various black-footed ferret sperm parameters across diets.

2.10a. Influence of breeding year and diet on sperm motility

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	40.36	6.18	6.53	**1.8e-08**
Diet: Carcass	-13.18	6.83	-1.93	**0.0587**
Diet: First-gen vitamin E	8.30	4.59	1.81	**0.0757**
Diet: Second-gen vitamin E	-0.172	5.06	-0.03	0.9729
Diet: Wild	9.48	7.49	1.27	0.2107
Breeding year	4.22	1.86	2.27	**0.0272**

2.10b. Influence of breeding year and diet on sperm forward progression (0-5)

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	2.58	0.23	11.27	**3.09e-16**
Diet: Carcass	-0.89	0.25	-3.53	**0.0008**
Diet: First-gen vitamin E	0.13	0.17	0.77	0.4433
Diet: Second-gen vitamin E	-0.15	0.19	-0.79	0.4345
Diet: Wild	0.09	0.28	0.31	0.7615
Breeding year	0.11	0.07	1.64	0.1073

2.10c. Influence of breeding year and diet on percent normal sperm

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	4.28	7.57	0.57	0.5738
Diet: Carcass	7.98	8.37	0.95	0.3442
Diet: First-gen vitamin E	5.49	5.62	0.98	0.3330
Diet: Second-gen vitamin E	1.98	6.20	0.32	0.7510
Diet: Wild	31.43	9.17	3.43	**0.0011**
Breeding year	7.26	2.28	3.19	**0.0023**

2.10d. Influence of breeding year and diet on percent abnormal acrosome

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	37.44	7.76	4.82	**1.06e-05**
Diet: Carcass	-11.95	8.58	-1.39	0.1690
Diet: First-gen vitamin E	1.14	5.77	0.20	0.8438
Diet: Second-gen vitamin E	1.09	6.35	0.17	0.8643
Diet: Wild	-21.63	9.40	-2.30	**0.0250**
Breeding year	4.10	2.34	-1.75	**0.0846**

2.10e. Influence of breeding year and diet on percent sperm with bent midpiece

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	19.60	4.00	4.90	**8.14e-06**
Diet: Carcass	5.88	4.42	1.33	0.189
Diet: First-gen vitamin E	-5.54	2.97	-1.86	**0.0676**
Diet: Second-gen vitamin E	-2.68	3.28	-0.82	0.4175
Diet: Wild	-7.41	4.85	-1.53	0.1318
Breeding year	-2.81	1.21	-2.33	**0.0231**

2.10f. Influence of breeding year and diet on percent sperm with bent midpiece without droplet

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	5.11	1.52	3.37	**0.0014**
Diet: Carcass	3.92	1.68	2.34	**0.0228**
Diet: First-gen vitamin E	-1.88	1.13	-1.67	0.1004
Diet: Second-gen vitamin E	0.26	1.24	0.21	0.8326
Diet: Wild	1.32	1.84	0.72	0.4759
Breeding year	-0.73	0.46	-1.59	0.1178

2.10g. Influence of breeding year and diet on percent sperm with bent tail without droplet

Fixed effects	**Estimate**	**Standard Error**	**t-value**	**p-value**
(Intercept)	11.80	3.41	3.47	**0.0010**
Diet: Carcass	10.84	3.76	2.88	**0.0056**
Diet: First-gen vitamin E	-6.10	2.53	-2.41	**0.0191**
Diet: Second-gen vitamin E	-2.08	2.79	-0.75	0.4590
Diet: Wild	-1.90	4.13	-0.46	0.6468
Breeding year	-1.80	1.03	-1.76	**0.0846**

2.10h. Influence of breeding year and diet on percent sperm with proximal droplet

Fixed effects	**Estimate**	**Standard Error**	**t-value**	**p-value**
(Intercept)	4.66	1.72	2.71	**0.0088**
Diet: Carcass	-3.65	1.90	-1.92	**0.0592**
Diet: First-gen vitamin E	0.73	1.27	0.57	0.5679
Diet: Second-gen vitamin E	-0.83	1.40	-0.59	0.5548
Diet: Wild	2.39	2.08	1.15	0.2558
Breeding year	0.07	0.52	0.13	0.8944

2.10i. Influence of breeding year and diet on percent sperm with distal droplet

Fixed effects	**Estimate**	**Standard Error**	**t-value**	**p-value**
(Intercept)	10.67	5.48	1.95	**0.0562**
Diet: Carcass	-10.23	6.05	-1.69	**0.0962**
Diet: First-gen vitamin E	7.66	4.07	1.88	**0.0646**
Diet: Second-gen vitamin E	5.29	4.48	1.18	0.2424
Diet: Wild	1.05	6.63	0.16	0.8747
Breeding year	2.34	1.65	1.42	0.1606

2.10j. Influence of breeding year and diet on percent sperm with damaged apical ridge

Fixed effects	**Estimate**	**Standard Error**	**t-value**	**p-value**
(Intercept)	34.99	7.65	4.58	**2.56e-05**
Diet: Carcass	-12.04	8.45	-1.43	0.1596
Diet: First-gen vitamin E	1.51	5.68	0.27	0.7919
Diet: Second-gen vitamin E	1.07	6.26	0.17	0.8644
Diet: Wild	-20.69	9.27	-2.23	**0.0294**
Breeding year	-3.77	2.30	-1.64	0.1073

Table 2.11. Mean, SEM and range for various black-footed ferret sperm parameters across breeding year and diet based on linear model significance.

2.11a. Percent normal sperm across breeding year (including wild individuals) in individual analysis. Mean provided ± SEM with range.

	2018	**2019**	**2020**	**2021**
Normal sperm	14.9 ± 6.65[b] (0-59)	22.9 ± 3.23[b] (2-56)	57.5 ± 5.25[a] (51-73)	35.2 ± 3.27[a] (3-61)

2.11b. Sperm metrics across diet treatments (including wild individuals) in individual analysis. Mean provided ± SEM with range.

	Control	**First Gen Vit E**	**2nd Gen Vit E**	**Carcass**	**Wild**
Abnormal acrosome	25.5 ± 3.77[a] (6-62)	30.6 ± 4.70 [ab] (0-74)	23.4 ± 4.15[ab] (2-15)	14 ± 2.72[ab] (7-24)	3.5 ± 0.866[b] (2-5)
Bent tail without droplet	6.57 ±1.63[a] (0-30)	2.19 ± 0.418[b] (0-7)	3.08 ± 1.64[ab] (0-22)	17.6 ± 8.78[ab] (0-51) -	4.5 ± 1.55[ab] (1-8) -
Damaged apical ridge	24.0 ± 3.80[a] (4-60)	29.1 ± 4.54[ab] (0-70)	22.2 ± 3.99[ab] (2-51)	12.4 ± 3.22[ab] (5-24)	3 ± 1.15[b] (1-5)

2.5 | Discussion

Diet has a significant impact on the health, development and reproductive success in wildlife maintained *ex situ* (G. Williams, 1999). Specifically, diet can directly impact fertility metrics like sperm quality, but can also impair sperm quality in subsequent generations (Bodden et al., 2020; Fan et al., 2015). With the declining reproductive success in the *ex situ* ferret population following a change in diet, we theorized that diet may be an attributing factor. Previous studies have shown that the TOR diet has excess vitamin A levels (Santymire et al., 2015) which may suppress vitamin E levels leading to a reduced ability to fight off ROS (Mazzaro et al., 1995).

Oxidative stress can negatively impact fertility (Barati et al., 2020). For example, ROS damaging sperm may be responsible for 30-80% of infertility cases in men (Barati et al., 2020). Excess ROS in sperm can both attack the sperm membrane and cause morphological changes that prevent sperm from fertilizing an egg, and also attack DNA directly which can lead to apoptosis, reduced pregnancy rates and improper embryo development (Bui et al., 2018).

The previous diet study conducted on the *ex situ* ferret population investigated the effects of supplementing the TOR diet with vitamin E and whole carcass on male reproduction and did not find conclusive evidence that antioxidant supplementation was beneficial to sperm parameters (Santymire et al., 2015, 2020). The TOR diet supplemented with vitamin E actually had lower percent normal sperm than the other diet treatments without vitamin supplementation, but had similar levels of normal sperm when supplemented with a rat or hamster carcass as the other diets that had better sperm results, indicating the importance for carcass in the diet. This previous study was conducted over one breeding season and offspring were not evaluated. To build on this, our goal was to determine whether vitamin E supplementation or a simulated "wild" diet could reduce oxidative stress levels and improve sperm parameters and reproductive success over generations by evaluating both sperm metrics as well as sperm DNA damage.

We observed lower sperm DNA damage in second-generation vitamin E ferrets, which were ferrets born to one or both parents supplemented with vitamin E, compared to first-generation vitamin E ferrets. Studies have shown that changes in parental diets can lower sperm count in offspring and change sperm epigenetic signatures in just one generation, both which have implications for offspring health and reproduction (Crisóstomo et al., 2021; de Castro Barbosa et al., 2016). It is likely that transmission of alterations happens not only through direct maternal reprogramming during pregnancy, but by epigenetic alterations in spermatozoa which

are then passed to offspring (Aiken et al., 2016). A 2014 study in mice found that sperm motility and capacitation in F1 males whose fathers were fed a high fat diet were reduced compared to offspring from control diet treatment fathers (McPherson et al., 2014).

The reduced DNA damage in second-generation vitamin E ferrets did not correlate with an increase in percent whelped or number of kits born. However, we did observe a strong negative correlation with increasing DNA fragmentation and several sperm metrics that are correlated with fertility, including percent normal sperm, percent normal acrosome, percent tightly coiled tail and percent damaged apical ridge. It is well-established that DNA damage is correlated with other sperm metrics (Lewis et al., 2005; Velez de la Calle et al., 2008). This finding in our study validates the use of sperm DNA damage as a metric that can be used to evaluate sperm health and reproductive potential.

Our study also found that wild ferrets had significantly better morphological sperm parameters than the other diets, and that the carcass diet did not follow the trend of the wild diet as we had predicted it would. This may imply that eating a wild diet and living in the wild over generations improves sperm parameters, potentially epigenetically, but that simply changing one's diet during a lifetime may not have the same effects, as we did not observe any significant improvements in sperm metrics for first generation carcass-only and vitamin E-supplemented individuals. This result is consistent with a study in jaguars that found free-ranging males had higher levels of normal sperm and concentration than zoo animals (Morato et al., 2001). Other studies in the endangered Iberian lynx (*Lynx pardinus*) and the Namibian cheetah (*Acinonyx jubatus*) found that there were no differences between captive and free ranging individuals (and even that free ranging lynx had lower sperm motility indices than captives) (Crosier et al., 2007; Gañán et al., 2010).

Investigating vitamin levels across diets illuminated some inherent and diet-induced differences in how vitamins are physiologically processed in the ferret. First, we observed a sex effect with females having higher vitamin A levels than males across both the control and first-gen vitamin E diets in our paired analyses. Establishing a general baseline for expected serum vitamin A levels in males vs. females is important for understanding how changing levels can affect ferret health and reproduction, especially since the previous ferret diet study was conducted only in males (R M Santymire et al., 2015). A study in Florida free-ranging panthers (*Puma concolor coryi*) found that females had higher serum retinol concentrations than males (Dunbar et al., 1999), but the authors did not know of any physiological reason why they would be higher, especially given pregnancy was not found to impact circulating retinol in females. In our individual vitamin A serum analysis, we observed a year and age effect. The serum vitamin A levels from ferrets in 2021 were significantly lower than 2017. This may be attributed to the fact that the COVID-19 pandemic affected the composition of the standardized TOR diet used in our study. The 2021 lots of the TOR had a higher fat content because of limited horse muscle meat available. The amount of fat in the diet is related to vitamin E absorption, with higher fat content promoting more vitamin E absorption as evident by higher vitamin E serum levels (Jeanes et al., 2004). Here, lower levels of serum vitamin A in the higher-fat 2021 diet males may be due to the higher levels of vitamin E absorption antagonizing vitamin A absorption. The previous diet study found that the higher levels of vitamin A in TOR antagonize vitamin E absorption (Santymire et al., 2015), and this antagonism is well-established in the literature (Santymire et al., 2015; Swick et al., 1952). This shift in the TOR diet might also help explain why percent abnormal acrosome was significantly lower in 2021 versus 2018 and percent normal sperm significantly higher in 2021 versus 2018; the increased fat may have improved sperm

health. Additionally, we observed lower serum vitamin A levels in 3-year-old males compared to 1-year-old males. Interestingly, we observed a trend of declining serum vitamin A levels with lower variability as age increased.

Serum vitamin A levels significantly decreasing in both the male and female first-generation vitamin E diet treatments follows the finding that vitamin E supplementation can reduce the amount of vitamin A formed and stored in the liver (Swick et al., 1952). This may impact circulating vitamin A levels and explain why we observed a significant decrease. Liver function may also explain why we observed a decline in vitamin A as ferrets aged. Liver function declines with age (Schmucker, 2005) which may result in lower levels of vitamin A storage in the liver if older ferrets are receiving the vitamin E supplemented diet. High levels of vitamin E compete with vitamin A for binding sites on lipoproteins when being transported in the blood (Pellett et al., 1994; Tsuruoka et al., 2019). A study in dairy cows found that vitamin E supplementation significantly decreased beta carotene (a form of vitamin A) content in serum, liver, fat and muscle (Yang et al., 2002). Our results contrast somewhat with results from the earlier diet study in black-footed ferrets, where the investigators found that vitamin E supplementation to a control diet did not decrease vitamin A levels compared to the pre-diet treatment (Santymire et al., 2015). However, that study investigated changes in serum vitamin levels after only 6 months (within a breeding season), whereas our sampling spanned several years.

When examining the effects of vitamin E supplementation, we did not observe a difference between sexes in either the control diet or first-generation vitamin E diet groups. This differs from results in other studies. For example, in a diet study on cheetahs, females had lower levels of vitamin E and selenium after controlling for diet treatment (Bechert et al., 2002). The

authors hypothesize that if females have higher levels of fat deposition, this would increase oxidative defenses, leading to lower circulating concentrations of antioxidants. In the ferrets, males are significantly larger than females (Miller et al., 1996), but differences in fat composition between sexes are not known. In many female mammals, fat composition is higher than that of males. If their fat composition is similar, perhaps both sexes metabolize vitamin E at the same rate.

Our results also validated our vitamin E supplementation methods since serum vitamin E levels increased in the first-gen vitamin E diet group. Several studies confirm that dietary supplementation of vitamin E increases vitamin E serum levels (Horwitt et al., 1972; Lehmann et al., 1977; Willett et al., 1983). In a previous ferret diet study, vitamin E supplementation also significantly increased after the treatment period (Santymire et al., 2015). These combined results serve as a validation that dietary vitamin E supplementation had a significant impact on circulating antioxidant levels in the first-generation vitamin E diet group.

In the individual vitamin E serum analysis, there were no factors that affected serum vitamin E levels, indicating that on average, vitamin E levels between control and vitamin E ferrets did not differ significantly. These results in conjunction with the paired analysis indicate that although vitamin E levels did significantly increase during supplementation, on average, the vitamin E levels for first- and second-generation vitamin E ferrets were not significantly higher than ferrets on the control diet. This might indicate that vitamin E was being absorbed at a similar rate by all ferrets. In a vitamin E supplementation experiment on cattle, vitamin E serum levels did not significantly differ between control and vitamin E pasture-fed cattle, which might indicate that the pasture diet had high levels of vitamin E as well, and that the intestinal absorption of tocopherols was limited or that plasma lipoproteins, which act as carriers for alpha-

tocopherol, were saturated (Yang et al., 2002). It is possible that plasma lipoproteins in both the control and vitamin E treatments were saturated for the ferrets. Additionally, intestinal absorption of vitamin E is facilitated by fat content in the diet (Traber, 2007). Although there were six 2021 ferrets included in this dataset, which implies that there was potentially a higher fat content for those individuals given the COVID-19 changes to the diet, the majority of the individuals included in the dataset theoretically received the same fat content in their diets. This may explain how serum vitamin E levels were the same across the diet treatments i.e. the rate of absorption was the same.

We did not observe an impact of the vitamin E supplementation on the ability to fight off oxidative stress via the proxy of serum TAC in our paired analysis. This is not an uncommon observation for long-term diet studies. Spikes in serum TAC levels are most often observed when evaluated several hours after diet intervention (Serafini & Del Rio, 2004). “Chronic” studies are more variable; many studies find no significant increase in TAC levels after several weeks of a dietary treatment, even when other antioxidants were shown to increase (Castenmiller et al., 1999; Miller et al., 1993; Young et al., 1999). For example, a two-week study that had participants eating a high antioxidant diet showed no significant increase in TAC or vitamin E levels even though ascorbic acid and vitamin A levels were increased (Record et al., 2001). Not observing significantly higher serum TAC levels in the vitamin E treatment individuals may also indicate antioxidant capacity is under homeostatic control and that only short-term consumption of high levels of antioxidants will make an impact (Record et al., 2001).

Interestingly, we observed a decrease in serum TAC levels in second generation vitamin E males compared to the first-generation vitamin E males in our individual analysis. This finding may indicate that second generation males had less demand for oxidative repair since their diet

incorporated more antioxidants, and therefore had less total antioxidant capacity overall. A mechanism of antioxidant mobilization posited by Elsayed (2001) suggests that antioxidant levels increase in the lungs and other organs in response to increased oxidative stress (Elsayed, 2001). Therefore, it would follow that if second-generation males had less oxidative stress, they have lower levels of circulating antioxidants. Additionally, a high fat diet (HFD) study in rats showed higher levels of TAC in serum for individuals on the HFD, indicating a mechanism that exists to match high oxidative stress levels with high levels of antioxidant activity (Maciejczyk et al., 2018). Since we observed both lower serum vitamin E levels and TAC in second-generation ferrets, this may suggest lower levels of oxidative stress levels. Additionally, recent work by Wang et al. (2001) has shown that 50% of the phenotypic variance in plasma TAC in humans is genetically controlled. It could be possible that ferrets born on the vitamin E diet had less oxidative stress, and therefore down-regulated genes that control TAC expression, causing them to express lower levels.

Seminal TAC levels (like serum TAC) did not correlate with fertility metrics, in contrast to other studies that found TAC levels were lower in infertile patients and were correlated with reduced sperm metrics (Mahfouz et al., 2009; Pahune et al., 2013). However, in evaluating DNA damage levels, there did exist an interaction between diet and TAC seminal plasma, which suggests that TAC in seminal plasma may vary with diet and thereby influence DNA damage levels in sperm. Alternatively, diet manipulation may not have significant impact on TAC levels, as one study found that seminal TAC levels may be regulated by systemic hormones such as prolactin (Mancini et al., 2009). While seminal TAC levels and traditional semen parameters were not correlated, we did observe a negative correlation between DNA damage and seminal TAC levels; low levels of TAC were more associated with higher levels of DNA damage and

vice versa. This result may indicate that the higher the ability of seminal fluid to protect against ROS, the less DNA damage will occur. Higher levels of ROS can induce single and double stranded DNA breaks in sperm (Aitken & Krausz, 2001). Other studies have observed a positive relationship between increased ROS and sperm DNA damage (Agarwal et al., 2002; Barroso et al., 2000; Shamsi et al., 2009). For example, a study in infertile men found those that had higher levels of ROS-TAC seminal fluid scores saw a significant increase in sperm DNA damage levels (Pahune et al., 2013). Another study in horses found a negative correlation between seminal plasma TAC and sperm DNA damage (Wnuk et al., 2010).

Our finding that TAC serum was positively correlated with sperm DNA damage is somewhat contradictory to the above results. This finding would suggest that having higher levels of antioxidants in blood serum were correlated with higher levels of sperm DNA damage. This result also contradicts our finding that higher antioxidant levels in seminal fluid are correlated with lower levels of DNA damage. However, our results show us that the lowest levels of DNA damage came from second generation vitamin E individuals, who also happened to have had the lowest serum TAC scores. Therefore, the correlation plot here may simply be presenting two separate results and not necessarily suggesting causation (i.e. that higher serum TAC causes higher sperm DNA damage). Additionally, the linear model did not find a significant interaction between TAC serum levels and diet in predicting DNA damage, but instead found that breeding year was significant (Fig. 7). This may indicate the correlation trend we see is more driven by breeding year than serum TAC levels in relation to DNA damage. It is also critical to remember that in this analysis, there were 19 data points, which also suggests that the sample sizes may be too small to parse out the confounding variables and truly uncover the driving factors and interactions between DNA damage, serum TAC levels and diet.

We also found that serum TAC and seminal fluid TAC were not correlated variables, which may suggest that TAC seminal fluid is not influenced by diet. Our study suggests that TAC serum was influenced by diet since second-generation vitamin E individuals had lower TAC serum, but it is possible that TAC seminal fluid levels are more highly genetically regulated than TAC serum regardless of diet to protect sperm from DNA damage. We have not found studies that examined fluid TAC levels subjected to different diets, but we postulate that seminal fluid TAC is regulated either genetically, hormonally, or a combination of these and other mechanisms to protect sperm from DNA damage regardless of changing environmental factors.

In conclusion, our vitamin E supplementation intervention did successfully increase circulating vitamin E levels in ferrets, which resulted in a lowering of vitamin A levels and may have downstream implications for oxidative stress regulation in the ferret. We found that serum oxidative stress levels decreased for second-generation vitamin E individuals, evidenced by lower levels of TAC as well as lower levels of sperm DNA damage. We also confirmed the relationship between DNA damage and traditional sperm parameters by finding that lower sperm DNA damage levels indicated higher percent motility, normal sperm and acrosome integrity. Our finding that seminal fluid TAC levels are negatively correlated with sperm DNA damage implies and confirms the protective quality of antioxidants to sperm DNA, regardless of whether they are dietarily or genetically regulated.

Wild ferrets continue to exhibit improved sperm metrics, indicating that several aspects of their natural environment, diet included, give a reproductive advantage over *ex situ* individuals. We plan to evaluate second-generation carcass individuals in the future to determine whether oxidative stress levels exhibit a similar declining trend as second-generation vitamin E individuals. Our study confirms that continuing ferrets on an antioxidant diet may have long-

term reproductive implications for the population overall. Our results may indicate that the benefits with DNA damage and TAC we see from vitamin E supplementation are transgenerational, since we do not see the same impact in first-gen vitamin E ferrets. Other studies have found that significant effects on reproduction in subsequent generation can take place due to maternal diet manipulation. For example, a study in rats found that a low protein diet in grandmothers with no diet manipulation in daughters caused lower ovarian reserve and higher levels of oxidative stress in ovarian tissue in granddaughters (Aiken et al., 2015). This impact may be due to direct exposure of the diet to the germ cells present in granddaughters, and a similar mechanism may be driving the effects we are seeing in the ferrets.

However, it is also possible that the positive impact vitamin E supplementation has on second-generation ferrets is conferred not through transgenerational programming via the maternal germline, but rather because second-generation ferrets are being fed vitamin E during early development. This is in line with the silver spoon effect, which posits that beneficial developmental conditions positively impact fitness in adulthood (Graffen, 1988). A 2020 study in *Drosophila* found that a low-yeast or high-sugar diet (both suboptimal, imbalanced diet types that have been shown to cause developmental and fertility issues) eaten during development lowered reproductive performance later in life, regardless of diet eaten in adulthood (Klepsatel et al., 2020). To test whether this mechanism is at play in the second-gen vitamin E ferrets, future researchers can create a separate group of ferrets who receive vitamin E supplementation immediately after weaning, but whose parents were both on the control diet.

One limitation of our study was that we were unable to sample captive individuals in 2020 due to the COVID-19 pandemic; this adds a gap in our diet study and did not allow us to evaluate individuals on a strict year-by-year basis. Additionally, the pandemic caused a shift in

the meat availability for the TOR diet, which may have significantly impacted our control diet and therefore the results and some conclusions we are drawing. Coincidentally, we may have discovered that a higher fat content in the TOR diet improved sperm parameters overall. Additionally, to perfectly mirror the wild diet in captivity, prairie dog meat is preferable to rat or hamster, but the availability of such meat is limited and therefore the wild diet cannot be perfectly re-created in captivity. It could be that rat and hamsters are less nutritionally valuable than wild prey since they've been bred over generations having been fed artificial diets in captivity, or that they are just generally leaner and have different nutritional composition.

Given the varying results we observed regarding vitamin E and A absorption across age and sex, it would be interesting to see the impact that the microbiome has across multiple diets. Researchers are elucidating more and more the role that the diet has on the microbiome and the role that gut microbiota play on vitamin absorption and overall health (David et al., 2014). Additionally, the etiology behind infertility has necessitated deeper levels of investigation, such as transcriptomic and genetic analysis. Transcriptomic analysis can pinpoint expression differences across key genes that differentiate fertile and infertile individuals (Prakash et al., 2021), helping to establish fertility biomarkers and elucidate the mechanisms behind infertility.

CHAPTER THREE | Characterizing the black-footed ferret sperm transcriptome: Investigating gene expression patterns across diet to uncover the mechanisms behind differing fertility parameters

3.1 | Abstract

Unique gene expression patterns in spermatozoa (sperm) can correspond to varying fertility phenotypes. We use the captive black-footed ferret (ferret; *Mustela nigripes*), which are experiencing a decline in fertility metrics potentially related to their oxidative-stress inducing diet, as one of the first, non-model endangered species to investigate the impact of the environment on the sperm transcriptome. We sequenced the transcriptome of ferrets across three major diet treatments: control: horsemeat + carcass, control + vitamin E supplementation + carcass, and wild ferrets eating natural prey, prairie dogs (*Cynomys* spp.), and conducted differential expression analysis and DAVID functional enrichment to understand which types of gene functions were enhanced between diets. We found that wild ferrets have enrichment in various microtubule- and olfaction-related functions, which may be related to the increased sperm health metrics we observe in wild ferrets generally. We also detected that *SMARCAD1,* a transcript involved in chromatin repair, was upregulated in vitamin E ferrets compared to controls, which may translate to the fact that second-generation vitamin E ferrets have significantly lower levels of sperm DNA damage than first-generation vitamin E ferrets (see Chapter Two). We also found that gene expression patterns in mature sperm across diets were not as different as we hypothesized. Understanding how sperm mRNAs differ across reproductive success can help us determine what gene expression patterns contribute to improved fertility metrics in this critically endangered species.

This chapter was a collaborative effort. Dr. Sergei Kliver (Saint Petersburg State University) conducted the genome assembly and annotation to which we aligned the

transcriptome; Dr. Manny Vazquez (University of California, Berkeley) supported lab work, conducted quality control on raw sequence data, trimmed and aligned the transcriptome, and conducted read counts; Dr. Olga Amelkina (Smithsonian Conservation Biology Institute) conducted differential expression and function enrichment analyses, and made the figures included in this chapter; Dr. Klaus-Peter Koepfli and Dr. Henrique Figueiró (Smithsonian-Mason School of Conservation) conducted BLAST searches for missing genes. The conception of this project, approach, sample collection, lab work, interpretation of analyses and writing were conducted by the author of this dissertation.

3.2 | Introduction

Selection pressures are circumstances that impact an organism's reproduction and survival, and these pressures will differentially impact fitness based on an individual's genotype (Papentin, 1973). In the era of the Anthropocene, these pressures can become so powerful that they overwhelm a species altogether, thereby rendering them threatened, endangered, or even extinct. The history of the black-footed ferret (ferret; *Mustelid nigripes*) exemplifies such a case. The ferret is North America's only endemic ferret species and one of the world's most endangered mammals. The spread of settlers across the American West in the early 1900s destroyed the ferrets' natural prairie habitats and decimated prairie dog populations (genus *Cynomys*), which ferrets rely on as prey (Dobson & Lyles, 2000). As a result, the ferret was brought to near-extinction. In the 1980s, the United States Fish and Wildlife Service (USFWS) brought the last 18 remaining ferrets into captivity in an effort to save the species via an *ex-situ* breeding and reintroduction program (B. Miller et al., 1996).

Seven ferrets of this group became the founders for the entire population that exists today. Since no new genes have been introduced into the population, relatedness between individuals is high and ferrets are suffering from symptoms of inbreeding depression (mean inbreeding in 2021 was F = 0.1356) (Marinari & Lynch, 2021). After more than 30 years of breeding, fertility rates in captive ferrets have plummeted, with pregnancy rates dropping from 60% in the 1990s to 46% in 2021 (Marinari, 2021). Additionally, captive male ferrets are experiencing a steady decline in normal sperm quality, dropping from 50% in 1990 to 35% in 2021 (Santymire et al. 2019; Santymire, pers. Comm.). The percent of morphologically normal sperm in an ejaculate is highly correlated with fertility (Brugh & Lipshultz, 2004; Kumar & Singh, 2015; Pukazhenthi et al., 2006), and the decline in ferret whelping rates correlates with this decline in normal sperm (Santymire et al., 2019). Wild ferrets, who were either reintroduced into the wild or were born in the wild and descended from reintroduced ferrets, stem from the same gene pool as captives but have nearly double (57.5%) the percent normal sperm than captives in 2020 (Santymire, pers. Comm.).

While captive and wild ferrets are genetically similar (Wisely, Buskirk, et al., 2002), the fitness outcomes, based on the different selective pressures they face, are drastically different. It seems that the *ex situ* environment may be negatively affecting ferret reproductive success (Santymire et al., 2021). This may be a case of Environmental-dependent Inbreeding Depression (EDID). EDID theory posits that only under certain environments will deleterious mutations cause a serious impact on fitness. In other words, deleterious mutations may not negatively impact an organism if the environment does not exacerbate it. EDID suggests that inbreeding depression has variable magnitudes based on what environmental condition is encountered (Cheptou & Donohue, 2011; Szulkin & Sheldon, 2007). If all ferrets share a similar genotype but

only some are encountering a serious decline in fertility due to environmental circumstances, EDID may be what the ferrets are experiencing.

One major difference between captive and wild ferrets is their diet. Wild ferrets eat prairie dogs along with other small rodents (B. Miller et al., 1996), whereas captive ferrets are fed a standardized, commercially-produced small carnivore diet (TOR; Milliken Meats Products, Lid, Markham, Ontario, Canada) along with whole carcass (usually rat, mouse or hamster). A decline in captive male ejaculate health began the year after all ferrets were switched to this TOR diet. Previously, their diet was composed of 60% mink pellets and 40% rabbit meat and made at each facility individually before it was decided that a standardized diet should be implemented across the SSP (Santymire et al. 2004). Diet can cause physiological, morphological and biochemical adaptations (Q. Liu et al., 2021). For example, before carcasses were included in their diet, skulls of captive ferrets were significantly smaller than those from wild ferrets (Wisely, Ososky, et al., 2002). Therefore, it is likely that the skulls of captive ferrets changed in response to the soft diet, a pattern observed in many other wild species taken into captivity (Hartstone-Rose et al., 2014).

The TOR diet is composed of horse meat and is high in polyunsaturated fatty acids (PUFAs) and vitamin A. High levels of vitamin A can prevent antioxidant activity, which can lead to oxidative stress (Agarwal & Saleh, 2002). Oxidative stress occurs when reactive oxygen species (ROS) overwhelm the antioxidant defense mechanisms in a system (Sies et al., 2017). ROS are oxidative radicals that are byproducts of metabolism and can also be exogenously introduced. They are essential to many biological and cellular pathways (Thomas, 2000; Tremellen, 2008; Wright et al., 2014). If a system cannot balance out ROS through antioxidant activity, oxidative damage may result. For example, sperm cell membranes, which are composed

largely of PUFAs, are vulnerable to lipid peroxidation, which is a cascade of oxidative attacks on the lipids that make up the membrane. Additionally, if a diet is high in PUFAs, sperm PUFA concentration can increase (Van Tran et al., 2017), making sperm even more susceptible to damage in the presence of oxidative stress. This can impact sperm morphology, sperm viability, sperm motility, DNA integrity and thereby fertility (Agarwal et al., 2002).

It is possible that the captive and wild diets may be causing differential gene expression in germ cells, thereby impacting the fertility phenotypes we observe. Changes in diet have been shown to exert influence on gene expression in gametes in other species, thereby impacting phenotypes. One study found that a chronic high-fat diet in mice led to increased histone acetylation in developing sperm, which downstream caused a decrease in SIRT6 protein, which is potentially involved in protamination processes (Palmer et al., 2011). Males with aberrant protamination have significantly decreased fertility (D. Miller et al., 2010). In another study, mice fed a low protein diet had global hypomethylation in epididymal sperm DNA, though there were no changes seen in sperm quality. However, females mated with the low protein diet males had decreases in uterine expression of several key genes involved in the inflammatory response and maternal recognition of pregnancy, which can impact pregnancy (Watkins et al., 2018).

The advantage of studying developing sperm is that they undergo gene expression changes as they move through different sections of the epididymis (Sullivan & Mieusset, 2016). Epididymal sperm are transcriptionally and translationally active. On the other hand, mature, ejaculated sperm are transcriptionally and translationally silent (Sullivan et al., 2016). However, working in an endangered species makes obtaining epididymal sperm problematic, since it requires removing the testes which thereby removes a breeder from the already small breeding

population. But, mature sperm can be collected via electroejaculation during the breeding season and does not disrupt their ability to mate (Howard, 1993).

Although mature sperm are transcriptionally inactive, the mature sperm transcriptome is richer than previously thought. Until recently, it was believed that RNAs present in mature sperm were useless relics left over from spermatogenesis (Hosken & Hodgson, 2014). While the majority of RNAs present in mature sperm are also present in testes, indicating that they indeed are produced during spermatogenesis, they are not simply relics (Sendler et al., 2013). The role of many of these RNAs is still unknown, but many of these transcripts have been found to contribute directly to fertility and embryo development (Hosken et al., 2014; Jodar et al., 2013). In humans, for example, differential expression in spermatozoa between fertile and infertile patients has proven that specific RNAs are critical to fertilization capacity (Ostermeier et al., 2004). Mature sperm contains both coding and non-coding (nc) RNAs, including mRNAs, siRNAs, miRNAs, piRNAs, and long ncRNAs (Jodar et al., 2013). The quantity of RNA found in human sperm is approximately 200 times less than the amount of RNA found in somatic cells (Jodar et al., 2013).

It is clear that some transcripts present in mature sperm are leftovers from spermatozoa development, such as the protamine transcript that codes for proper DNA compaction. These transcripts are not useful in mature sperm, since DNA has already been compacted during spermatogenesis. However, there are a number of RNAs whose production markedly increases at the latter stages of spermatogenesis (Sendler et al., 2013). It is likely that this boost in production of RNA transcripts allows the RNAs to subsist in large quantities until after sperm maturation, where they play a role in fertility and embryo development. For example, some of these RNAs were found to be likely related to early embryonic growth and biological rhythm (Sendler et al.,

2013). Another study across four mammal species found that mRNAs present in mature sperm are translated into proteins in the female reproductive tract that assist in sperm capacitation, the physiological changes that take place to allow for acrosomal penetration into the ovum (Gur & Breitbart, 2006).

By investigating gene expression in mature sperm across different diets in ferrets, my goal was to determine whether there exist signatures of upregulation in antioxidant defense pathways to ward off oxidative stress potentially caused by the captive diet. I also investigated whether key fertility genes are differentially expressed across diets, indicating that the nutritional stress from certain foods is impacting reproductive success directly or indirectly. Testing for up or downregulation in such genes might indicate that these gene expression changes may have taken place during sperm development in response to the dietary conditions that ferrets are experiencing. Additionally, by uncovering the sperm transcriptome of wild ferrets, I can better understand the natural gene expression patterns in the species. By establishing this baseline of expression, I can explore how captive ferrets on different diets diverge, providing insight into which diet most reflects the wild expression patterns, particularly in key fertility genes. This work is one of the first studies that examines sperm cell transcriptomics in a non-model, endangered species.

3.3 | Methods

3.3.1 | Dietary treatments

Captive individuals in this study were on one of three dietary treatments (Table 3.1). At the USFWS National Black-Footed Ferret Conservation Center (FCC) in Carr, Colorado, 10 males were on a horsemeat diet + prey item (2 per week; rat or hamster pieces), which served as

the control, and 15 males were on horsemeat diet + vitamin E (D-α-tocopherol; 400 IU/kg dry matter basis) + prey item (2 per week), which served as the vitamin E supplemented diet. Of these 15 individuals, 2 were first-generation ferrets (meaning they started the vitamin E diet during their lifetime after having been on the control diet previously) and 13 were second-generation ferrets (meaning at least one parent was on a vitamin E diet, and that they were fed a vitamin E diet throughout their lives). These two generations were combined due to the small sample size of first-generation vitamin E ferrets. FCC also had two ferrets that were transferred from Louisville Zoological Gardens (LZG; Louisville, KY), where a carcass diet is being implemented (carcass of rat or mice only); this treatment emulates the wild diet. Three of the controls included in our sample are also from LZG. We also had samples from four wild individuals at Rocky Mountain Arsenal, Colorado, who were eating a wild diet (prairie dog). This study was approved by the LPZ Research Committee (proposal #2007–005).

Table 3.1. Number of individuals included in each diet treatment (N) and location where samples were collected.

Diet	N	Location
Control	10	FCC, LZG
Vitamin E	15 (13 second generation, 2 first generation)	FCC
Carcass	2	FCC
Wild	4	Rocky Mountain Arsenal

3.3.2 | Sample collection, processing & storage

For male ferrets, testes firmness is an indicator of sperm production and breeding readiness (Williams et al., 1991). Semen was collected from anesthetized males (Ketaject 38.8 mg/kg; diazepam 0.06 mg/kg) by electroejaculation (Howard, 1993) in March of 2020 for wild

ferrets and April of 2021 for captive ferrets, depending on testicular tumescence via palpation (R M Santymire et al., 2006, 2007). A rectal probe delivered electro-stimulations over a voltage range of 2-5, and semen was collected using a micropipette (Howard, 1993; Wolf et al., 2000). After semen collection, cells were evaluated for percent motility, motility status (forward progression of 0-5; 5 is the highest score), normal morphology and concentration. Acrosomal integrity was assessed using phase-contrast microscopy (1000×) on 100 sperm cells per sample, as described previously (Santymire et al., 2006; Wildt et al., 1989). For RNA-Seq analysis, 3-5 μl of semen was collected using a micropipette after electroejaculation and directly added to vials of RNA-later solution (Invitrogen). Samples were allowed to equilibrate at room temperature for 5 minutes and then directly frozen in liquid nitrogen or placed on dry ice for future use.

Wild ferrets (n = 4 males) were all wild-born and trapped in March 2020 at Rocky Mountain Arsenal, Colorado. The ferret reintroduction at this site began in 2015. Trapping and immobilization followed protocols of the Black-Footed Ferret Recovery Implementation Team (USFWS, 2016) and Kreeger et al. (1998). Briefly, animals were cage-trapped at night and returned to the same location following examination and recovery from anesthesia, usually within 1 hr of capture. All trapping was authorized by the USFWS under permit #TE064682-1 and was conducted by the U.S. Forest Service, National Park Service and Prairie Wildlife Research as part of routine population monitoring. All animal experiments conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Lincoln Park Zoo Research Committee (Chicago, IL) and USFWS (Carr, CO). Semen collection and storage followed the same procedures as above.

3.3.3 | RNA extraction, library prep, and sequencing

For RNA extraction, samples were thawed on ice. They were then washed with cold PBS and spun down in a centrifuge for 5 minutes at 1000 rpm to remove buffer and seminal fluid; this process was repeated 3 times. Cell lysis, cDNA conversion, fragmentation, and library prep were done in single tubes using the QIAseq FX Single Cell RNA Library Kit (Qiagen, USA). Final library amplification was performed using Q5 Hot Start polymerase (New England Bioscience), utilizing indexing primers and universal Illumina adapters obtained from the Functional Genomics Lab at the University of California, Berkeley (FGL). Final libraries, which were constructed for each individual sample, were submitted to the FGL for library quality check via Bioanalyzer (Agilent) and subsequent 150 bp paired-end sequencing on Illumina NovaSeq S4 flow cell, attempting 37 million reads per library.

3.3.4 | Sequenced data processing

Sequence data quality check was conducted using FastQC. Trimmotatic (Bolger et al., 2014) was used to remove adapter content, low quality reads, and artificial poly-G tails from all reads. The processed and filtered reads were then aligned to the chromosome-length genome assembly of the ferret (NCBI accession GCA_022355385.1) using STAR (Dobin et al., 2013). RSEM (B. Li & Dewey, 2011) was used to refine STAR-mapped reads into transcript level counts (transcripts per million, TPM).

3.3.5 | Differential expression analysis

Data visualization and differential expression analysis was performed on reads obtained from 31 samples using the DESeq2 R package (Love et al., 2014). For visualization, size factors

were estimated from the count data of all samples and the Relative Log Expression (RLE) normalization was used to obtain regularized log transformed values. These normalized values were then used for principal component analysis (plotPCA function in DESeq2 R package) and creation of clustered heatmaps (pheatmap R package). Differential expression analysis was performed on 29 samples from three diet groups (Control, Vitamin E, and Wild) with three pairwise comparisons: Control vs. Wild, Vitamin E vs Wild, and Vitamin E vs. Control. A Wald test was used on genes that passed an independent filtering step and resulting p-values were adjusted for multiple testing using Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995). Genes with absolute fold change $|fc| \geq 2$ and an adjusted P-value (q-value) < 0.05 as calculated by DESeq2 were considered differentially expressed.

3.3.6 | DAVID Enrichment analysis

Selected genes from differential expression analysis that passed conditions of p-value $<$ 0.05 and $|fc| \geq 2$ were used for gene-set functional enrichment analysis with DAVID tool (Huang et al., 2007), setting species to human. For each comparison pair, total number of genes and separately up- and downregulated genes were analyzed. EASE score (modified Fisher Exact p-value of enrichment) was set to 0.05. Datasets used for analysis included Gene Ontology (Consortium, 2004) and KEGG pathways (Kanehisa et al., 2017). Clustering tool in DAVID was used to unite redundant functional terms together for visualization.

3.3.7 | Breeding and Sperm Quality Analysis

One aim in this study was to determine whether fertility was different between diet groups, and if so, whether key genes involved in fertilization capacity were differentially

expressed. Therefore, we assessed reproductive outcomes for each individual where data were available, as well as sperm parameters (which are important predictors of fertility).

Reproductive data were collected by managers at the two *ex situ* facilities (FCC and LZG) and were subsequently combined and standardized in Python (Version 3, Van Rossum & Drake, 2009). Breeding records spanned 2004 until 2020, but were matched for the three years of the diet study (2017-2021) based on the year the RNA sperm sample was collected. Not all individuals were paired for reproduction and therefore, not all records have an associated breeding outcome. The 17 individuals from this study had reproductive outcomes (1 carcass, 7 controls, 9 vitamin E).

Breeding outcome was categorized as “whelped” if dams came to term and had kits, or “did not whelp” if dams did not produce kits. Total number of kits for all breeding outcomes were summed and also used as a reproductive metric. Linear models were used to evaluate whether reproductive outcomes were influenced by diet. A threshold of 0.05 was used as the p-value cutoff in the linear model to warrant post-hoc testing using a Kruskal-Wallis test. If the Kruskal-Wallis test had a p-value less than 0.05, apairwise Wilcoxon signed-rank tests were used to determine significant differences between the various groups.

Sperm parameters were evaluated as described earlier and recorded for each individual in this study. Linear models were used to evaluate whether sperm parameters were influenced by diet. A threshold of 0.05 was used as the p-value cutoff in the linear model to warrant post-hoc testing using a Kruskal-Wallis test. For p-values less than 0.05, Wilcoxon signed-rank test was used for pairwise comparison between the groups.

3.4 | Results

3.4.1 | Transcriptome profile across different diets

Sequence data from all samples passed the initial quality check. After trimming, ~ 98 % of reads mapped to one site, < 2 % of reads mapped to multiple sites, and 0 reads failed to map. A total of 17,295 genes were expressed at a detectable level.

Principal component analysis (PCA) was used to visualize the variation in data based on the overall normalized expression. Projection of the samples onto the first principal component explained 24% of the variability and projection onto the second principal component captured 20% of the variability (Fig. 3.1). The PCA revealed little variation in transcriptome expression profiles across samples, with no obvious clustering of animals based on the diet. Visualization using heatmap further supported this observation, with no clusters forming based on the diet or captive and wild status (Fig. 3.2).

Two individuals were more separated from the group than others - one control individual and one wild individual (Fig. 3.1 & 3.2) and accounted for the variances along PC1 and PC2. Further investigation into mean kinship, inbreeding coefficient, parental history, reproductive status, and sperm parameters (percent motility, normal sperm, abnormal acrosome, forward progression and concentration) do not seem to contribute to the observed variation, which may indicate that other biological factors are at play. All vitamin E, control and carcass samples were processed together, with wild samples being processed separately, but protocol conditions would not contribute to this variation since separated samples were processed with their counterparts (controls with controls, wild with wild, etc.). Additionally, sequencing quality was high amongst all samples and therefore, it is unlikely that sequencing error contributed to these separating from the group.

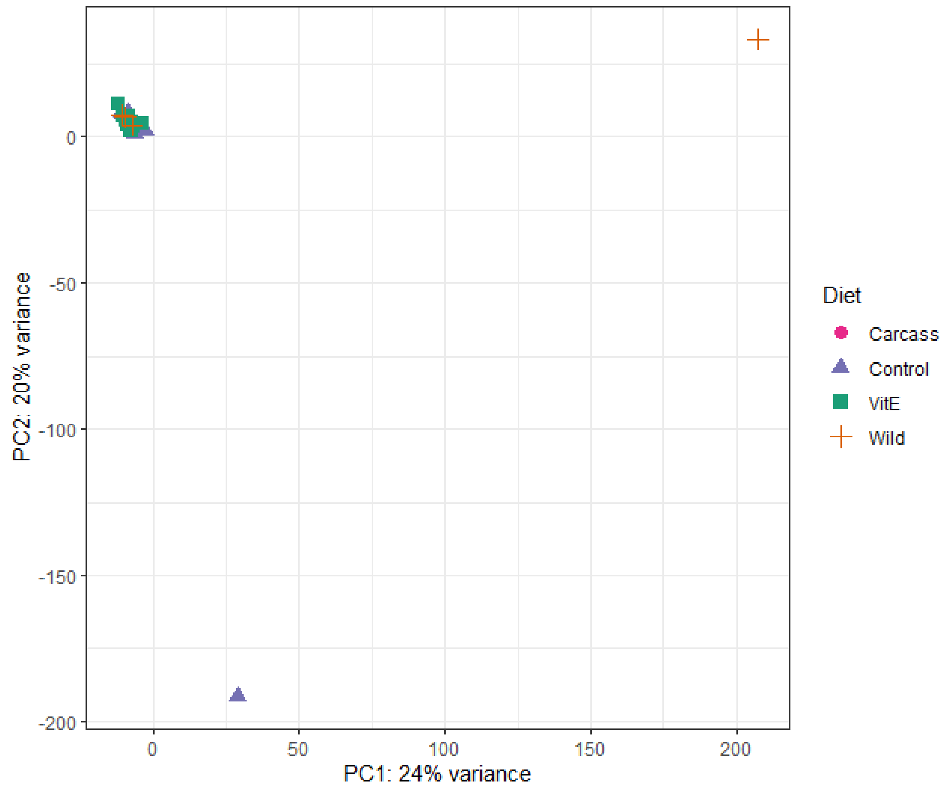

Figure 3.1. Principal component analysis plot representing variation in samples from all diet groups. Components 1 and 2 describe 44% of the total variance in the dataset.

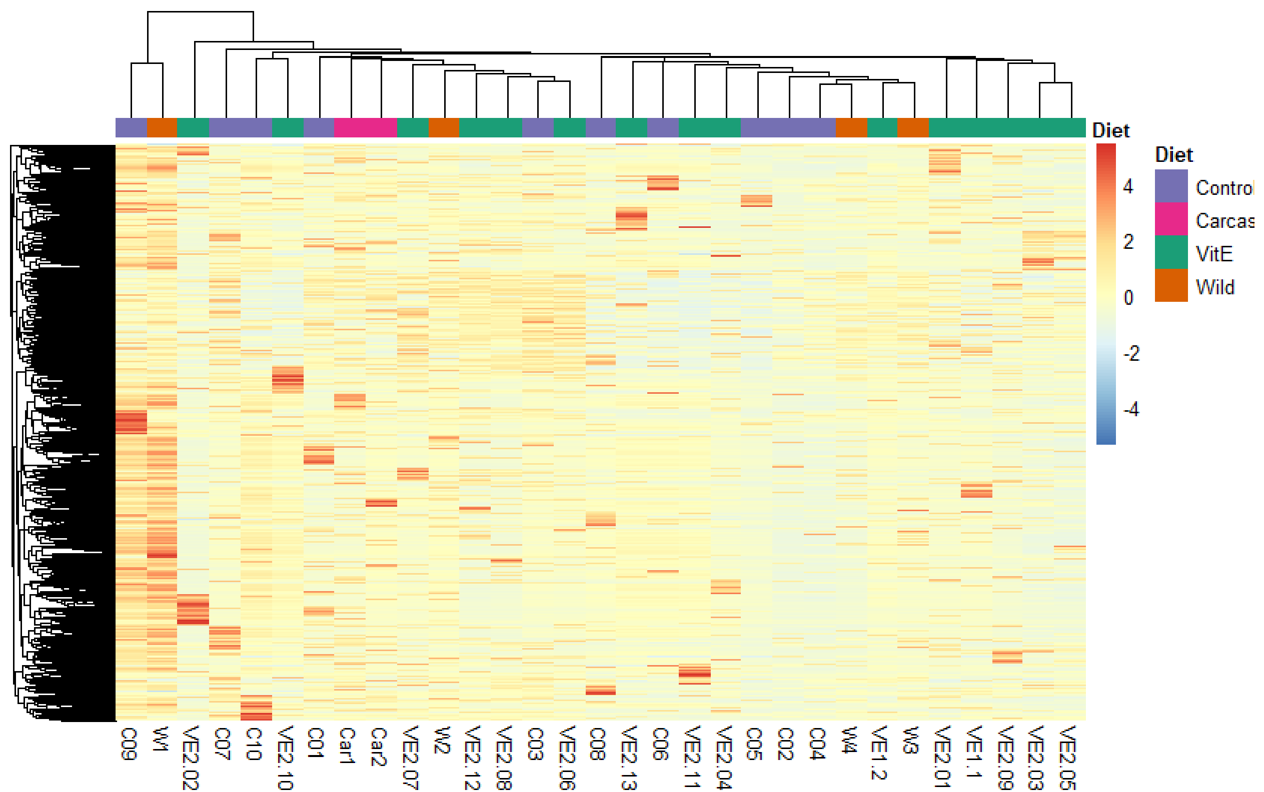

Figure 3.2. Heatmap of one-way hierarchical clustering analysis (Euclidean method, complete linkage) using Z-score for RLE normalized values of all genes expressed in ferret mature sperm from different diet groups.

3.4.2 | Differential gene expression analysis

Differential expression analysis revealed 318 genes satisfying conditions of $p < 0.05$ and $|fc| \geq 2$ in Control vs Wild pair (Fig. 3.3A), 130 genes in Vitamin E vs Wild pair (Fig. 3B), and 686 genes in Vitamin E vs Control pair (Fig. 3.3C). Out of these genes, 3 genes (LRRC37B, ZNF462, S100A10) were shared between all 3 comparison pairs, 77 genes (66 downregulated and 11 upregulated) were shared between Control vs Wild and Vitamin E vs Wild pairs, 16 genes were shared between Vitamin E vs Wild and Vitamin E vs Control pairs, and 126 genes were shared between Control vs Wild and Vitamin E vs Control pairs.

After correcting the p-value for false discovery rate (q-value < 0.05), 6 genes were left differentially expressed in Control vs Wild pair (Table 3.1, Fig. 3.3D), 4 genes in Vitamin E vs

Wild pair (Table 3.2, Fig. 3.3E), and 36 genes in Vitamin E vs Control pair (Table 3.3, Fig. 3.3F). No differentially expressed genes were shared between these comparison pairs.

Table 3.2. Differentially expressed genes in Control vs. Wild groups, satisfying conditions of q-value < 0.05 and |fc| ≥ 2. Indeterminate gene MUSNIGG00033377 was a short segment that showed multiple hits on different chromosomes or scaffolds of the domestic ferret and human genomes; it is likely a repetitive region.

Gene Symbol	Description	p-value	q-value	Fold Change
MUSNIGG00033377	Indeterminate	1.08E-05	0.03	-28.34
DNMT1	DNA methyltransferase 1	3.51E-05	0.05	205491.04
MRPS24	mitochondrial ribosomal protein S24	1.69E-05	0.03	238238.19
TRIM54	tripartite motif containing 54	1.79E-05	0.03	257694.53
HPCA	hippocalcin	1.39E-05	0.03	261290.69
PRR14	proline rich 14	1.31E-05	0.03	265511.37

Table 3.3. Genes that were differentially expressed in Vitamin E vs. Wild groups after a corrected p-value adjustment below 0.05 and fold change above 2 (n=6). Base mean represents normalized counts of all samples. Fold change represents the number of times the gene is expressed more or less in the Vitamin E group compared to the Wild group.

Gene Symbol	Description	p-value	q-value	Fold Change
RDH10	retinol dehydrogenase 10	2.36E-05	0.03	-387.71
PTX3	pentraxin 3	1.41E-05	0.03	-30.98
LRRC37B	leucine rich repeat containing 37B	4.40E-05	0.04	-17.07
HSD11B1L	hydroxysteroid 11-beta dehydrogenase 1 like	2.32E-05	0.03	524729.7 7

Table 3.4. Differentially expressed genes in Vitamin E vs. Control groups, satisfying conditions of q-value < 0.05 and |fc| ≥ 2.

Gene Symbol	Description	p-value	q-value	Fold Change
IGKV2-26	immunoglobulin kappa variable 2-26 (pseudogene)	5.83E-05	0.02	-51.64
CREBZF	CREB/ATF bZIP transcription factor	9.79E-07	0	-47.47
P2RX7	purinergic receptor P2X 7	1.00E-04	0.02	-36.16
USP15	ubiquitin specific peptidase 15	1.06E-04	0.02	-26.87
GPR55	G protein-coupled receptor 55	3.36E-04	0.05	-25.95
TNXB	tenascin XB	7.90E-06	0.01	-24.27
KCNJ5	potassium inwardly rectifying channel subfamily J member 5	2.29E-04	0.04	-22.58
ECM2	extracellular matrix protein 2	8.37E-06	0.01	-20.2
INTS2	integrator complex subunit 2	2.22E-05	0.02	-19.77
ITSN1	intersectin 1	1.07E-04	0.02	-19.64
GUCY1A3	Guanylate Cyclase 1 Soluble Subunit Alpha 1	1.20E-05	0.01	-19.61
UBOX5	U-box domain containing 5	8.02E-05	0.02	-18.56
UBOX5	U-box domain containing 5	8.02E-05	0.02	-18.56
MECOM	MDS1 and EVI1 complex locus	3.83E-07	0	-17.87
PLK1S1	Polo-Like Kinase 1 Substrate 1	7.01E-05	0.02	-14.84
ZNF879	zinc finger protein 879	6.42E-05	0.02	-14.41
KALRN	kalirin RhoGEF kinase	5.39E-05	0.02	-13.71
DTL	denticleless E3 ubiquitin protein ligase homolog	1.39E-04	0.03	-13.28
SERPINB8	serpin family B member 8	5.78E-05	0.02	-12.7
PTPRZ1	protein tyrosine phosphatase receptor type Z1	1.55E-05	0.01	-12.68
TNRC6C	trinucleotide repeat containing adaptor 6C	1.50E-04	0.03	-12.35
KPNA6	karyopherin subunit alpha 6	1.36E-04	0.03	-11.53
BORA	BORA aurora kinase A activator	5.97E-05	0.02	-10.71
TRPM3	transient receptor potential cation channel subfamily M member 3	2.28E-04	0.04	-10.62
HIVEP1	HIVEP zinc finger 1	2.16E-04	0.04	-9.28
SPAM1	sperm adhesion molecule 1	8.61E-05	0.02	-8.83
KCNJ3	potassium inwardly rectifying channel subfamily J member 3	1.11E-04	0.02	-8.69
REL	REL proto-oncogene, NF-kB subunit	2.95E-04	0.04	-8.66

Gene Symbol	Description	p-value	q-value	Fold Change
UTP18	UTP18 small subunit processome component	2.20E-04	0.04	-8.58
PCDH19	protocadherin 19	2.86E-04	0.04	-8.08
NF1	neurofibromin 1	3.64E-04	0.05	-7.87
ZNF804A	zinc finger protein 804A	1.55E-04	0.03	-7.32
KCNB2	potassium voltage-gated channel subfamily B member 2	4.40E-05	0.02	-6.47
TENM3	teneurin transmembrane protein 3	2.83E-04	0.04	-6.02
RAPGEF2	Rap guanine nucleotide exchange factor 2	3.02E-04	0.04	-4.85
LRRFIP1	LRR binding FLII interacting protein 1	5.12E-05	0.02	22.47

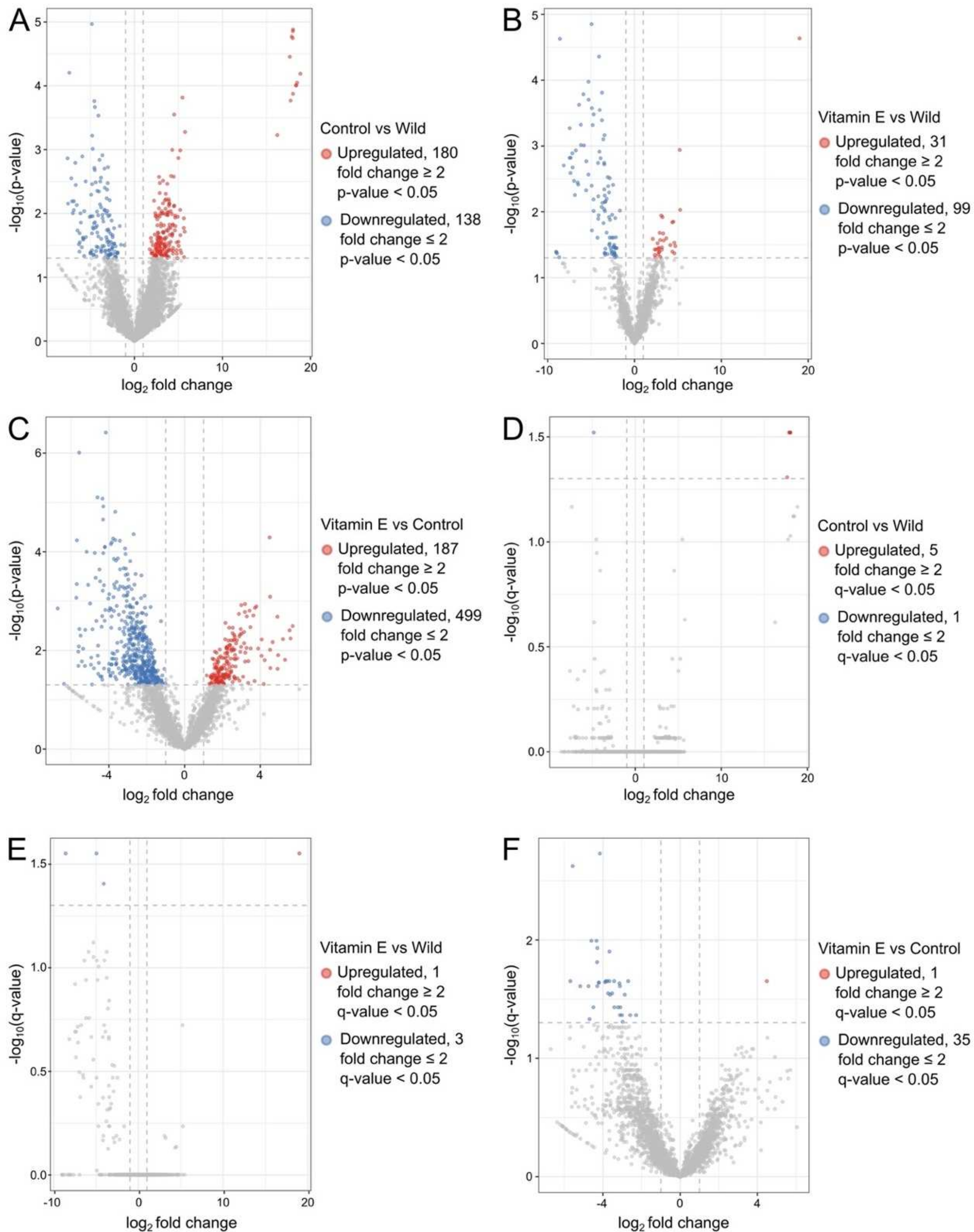

Figure 3.3. Volcano plots showing upregulated and downregulated genes. A, B & C plots use Wald test, p-value < 0.05), |fc| ≥ 2 ; D, E & F plots are corrected for false discovery rate. (**A, D)** Control vs Wild pair, (**B, E)** Vitamin E vs Wild pair, and (**C, F)** Vitamin E. vs. Control pair.

Table 3.5 Differentially expressed genes in Control vs. Wild groups, satisfying conditions of p-value < 0.05 and |fc| ≥ 2.

Gene Symbol	Description	p-value	q-value	Fold Change
PIAS2	Protein Inhibitor of activated STAT 2	6.47E-05	0.07	18.84
SLA2	Src like adaptor 2	8.95E-05	0.08	18.48
STPG1	sperm tail PG-rich repeat containing 1	9.78E-05	0.08	18.38
BOP1	BOP1 ribosomal biogenesis factor	9.87E-05	0.08	18.37
PRR14	proline rich 14	1.31E-05	0.03	18.02
PITPNM1	phosphatidylinositol transfer protein membrane associated 1	1.34E-04	0.09	18.02
HPCA	hippocalcin	1.39E-05	0.03	18.00
TRIM54	tripartite motif containing 54	1.79E-05	0.03	17.98
MRPS24	mitochondrial ribosomal protein S24	1.69E-05	0.03	17.86
DEFB124	defensin beta 124	1.70E-04	0.10	17.75
DNMT1	DNA methyltransferase 1	3.51E-05	0.05	17.65
HSD11B1L	hydroxysteroid 11-beta dehydrogenase 1 like	5.91E-04	0.24	16.24

3.4.3 | Functional enrichment in different diet groups

Selected genes from differential expression analysis satisfying conditions of $p < 0.05$ and $|fc| \geq 2$ were used for functional enrichment analysis.

There were 7 functional terms enriched in wild compared to control ferrets (with 3 of those falling into the cluster of olfactory transduction) (Fig. 3.4A) and 15 functional terms enriched in control compared to wild ferrets (with 3 of those falling into the cluster of extracellular matrix) (Fig. 3.4B). There were 8 functional terms enriched in wild compared to vitamin E individuals (with 3 of those falling into the cluster of microtubule-based movement)

(Fig. 3.4C) and 2 functional terms enriched in vitamin E compared to wild individuals (not pictured). There were 30 functional terms enriched in control compared to vitamin E individuals (4 of those in the cluster of olfactory transduction, 3 in the cluster of microtubule-based movement, 3 in the potassium channel cluster, 2 in GTPase activity cluster, and one each in mRNA processing body and peptide binding) (Fig. 3.5A) and 24 functional terms enriched in vitamin E compared to control individuals (5 of those in the cluster of GPCR signaling, 3 in the cluster of phagosome, 3 in the cluster of potassium channel, and 1 in focal adhesion) (Fig. 3.5B).

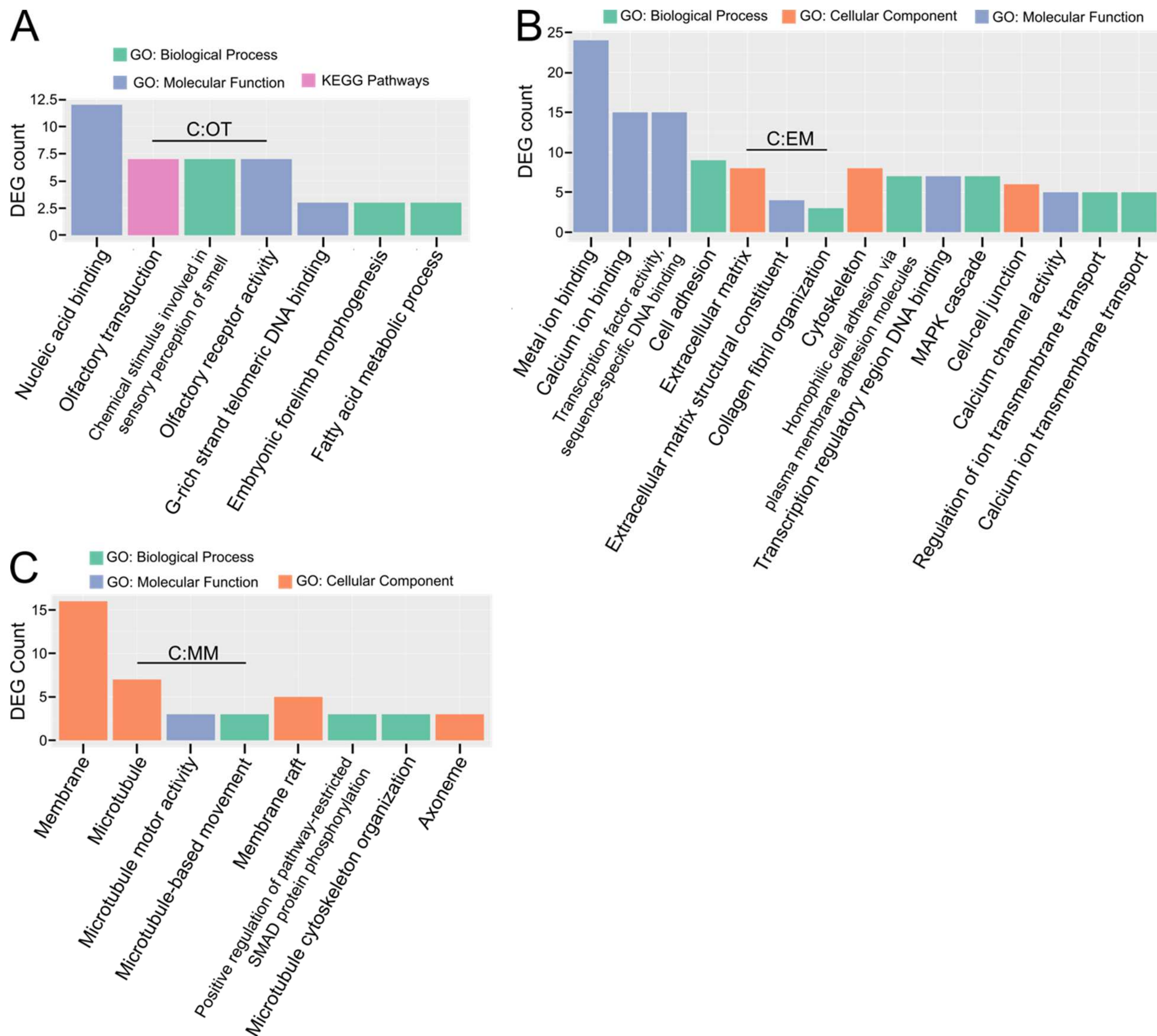

Figure 3.4. Functional terms and pathways enriched in sperm from different diet groups. Plots display enriched gene-sets based on selected genes from differential expression analysis that are **(A)** downregulated in Control vs Wild, **(B)** upregulated in Control vs Wild, **(C)** downregulated in Vitamin E vs Wild diet groups. C:OT, olfactory transduction cluster; C:EM, extracellular matrix cluster; C:MM, microtubule-based movement cluster.

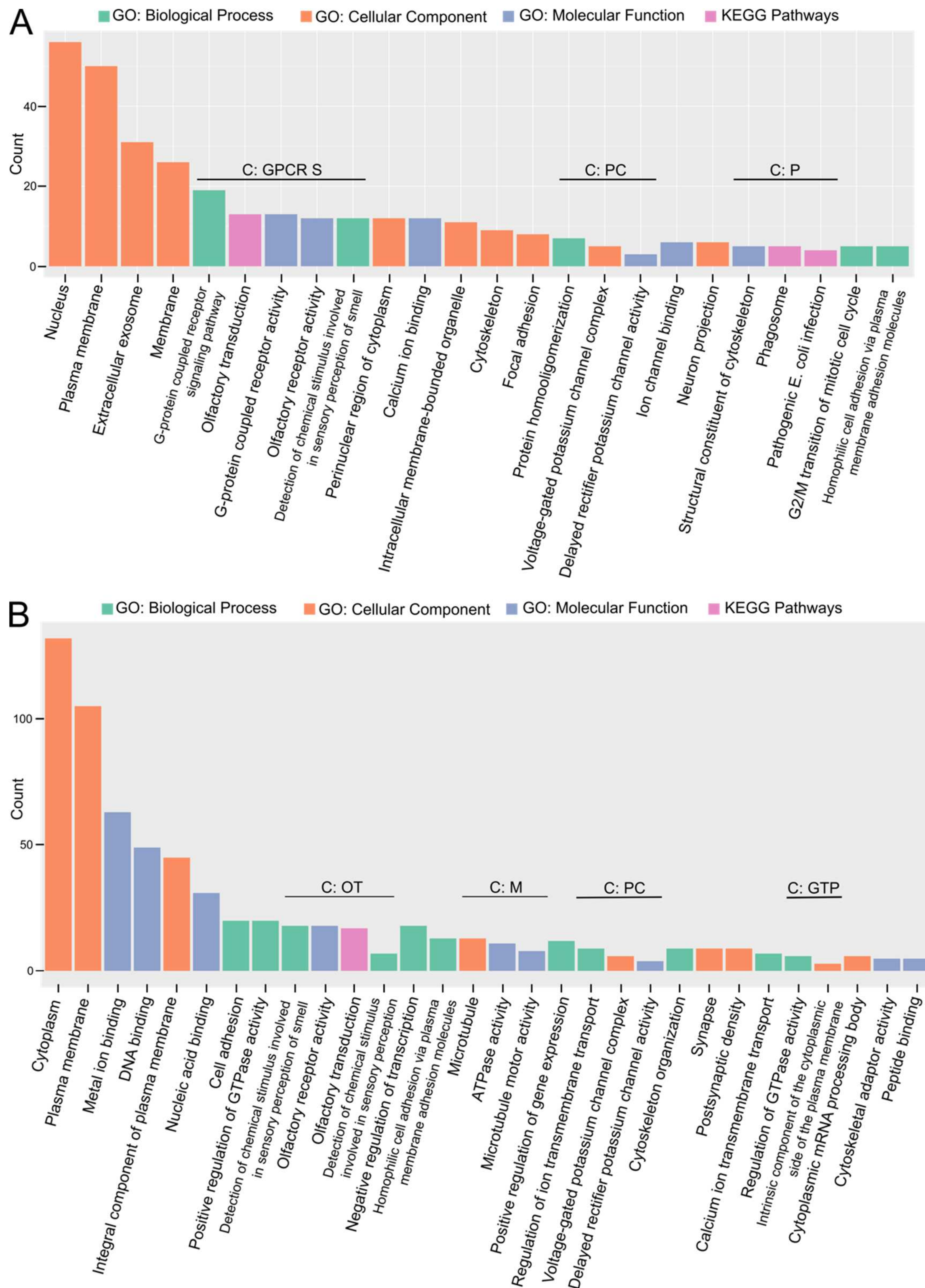

Figure 3.5. Functional terms and pathways enriched in sperm from different diet groups. Plots display enriched gene-sets based on selected genes from differential expression analysis that are **(A)** upregulated in Vitamin E vs. Control and **(B)** downregulated in Vitamin E vs. Control. C:GPCR S, GPCR signaling cluster; C:PC, potassium channel cluster; C:P, phagosome cluster; C:OT, olfactory transduction cluster; C:M, microtubule cluster; C:GTP, GTPase activity cluster.

Specifically, functional terms enriched in sperm cells between control vs. wild individuals (enriched in wild ferrets) were related to olfactory transduction (aiding in cellular changes and response to smell), nucleic acid binding (related to regulation of RNA and DNA transcription), G-rich strand telomeric DNA binding (having to do with telomere length regulation), embryonic forelimb morphogenesis, and fatty acid metabolism (Fig. 3.4A). Functional terms enriched in sperm cells between control vs. wild individuals (enriched in control ferrets) included ion binding and transport, extracellular matrix functions, transcription factor regulation and DNA binding, cell functions like adhesion and junctions, MAPK signaling processing, cytoskeleton function, and calcium channel (Fig. 3.4B).

Functional terms enriched in sperm cells between vitamin E vs. wild (enriched in wild ferrets) included membrane processes (such as budding, rafts, vesicles, transport, and transmembrane processes), microtubule movement, SMAD protein phosphorylation (directly related to cell development and growth), axoneme (related to cilia and flagella), and cytoskeleton function (Fig. 3.4C). Functional terms enriched in vitamin E compared to wild ferrets included nucleolus (contributing to internal cellular processes and proliferation) and negative regulation of type I interferon production (having to do with immune and inflammatory response).

Functional terms enriched in sperm cells between vitamin E vs. control individuals (enriched in vitamin E ferrets) using *Homo sapiens* as a reference included several cellular processes (like membrane functions, cytoplasm, cell adhesion, phagosome functions), GPCR signaling (related to transmembrane signaling), cytoskeleton, focal adhesion, ion binding, and potassium channel (Fig. 3.5A). Functional terms enriched in sperm cells between vitamin E vs. control individuals (enriched in control ferrets) using *Homo sapiens* as reference included ion binding and transport, cellular processes (like membrane functions, cell adhesion), olfactory

transduction, nucleic acid binding, potassium channel, GTPase activity (related to cellular movement), peptide binding, microtubule function and mRNA processing (Fig. 3.5B).

3.4.4 | Statistical analyses of reproduction and sperm parameters

Percent whelped and total number of kits were not different across diets. Additionally, percent motility, forward progression, percent abnormal acrosome and sperm concentration were not significantly different across diets. However, vitamin E impacted percent normal sperm ($p = 0.034$, Table 3.6). Upon post-hoc testing ($\chi^2_3 = 11.809$, $p < 0.05$), percent normal sperm was lower in vitamin E individuals compared to wild individuals ($p = 0.028$) (Fig. 3.6).

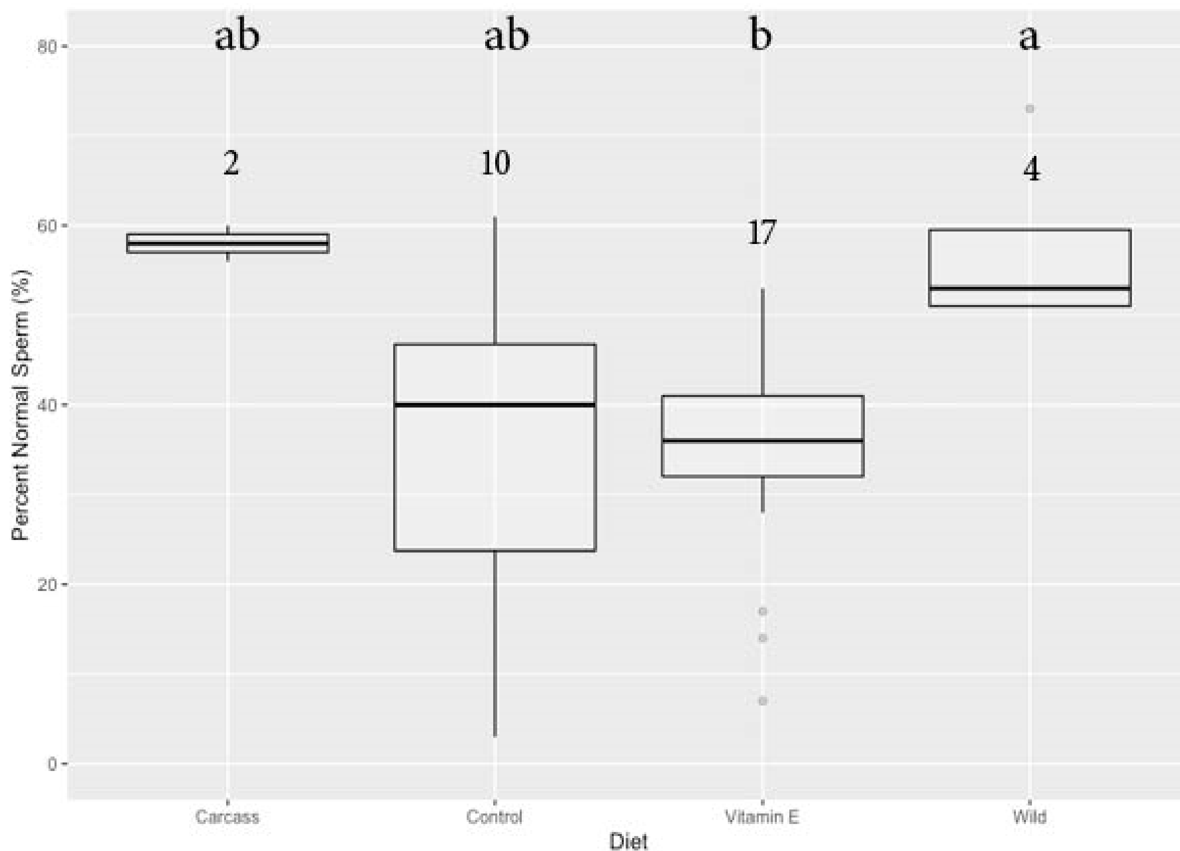

Figure 3.6. Percent normal sperm morphology (NSM) by diet. Number above bars is the number of ferrets included in that treatment. Superscripts indicate differences ($p < 0.05$) in percent NSM between diet groups.

Table 3.6. Model parameters from the fitted linear model testing the influence of diet on percent normal sperm morphology.

Fixed effects	Estimate	Standard Error	t-value	p-value
(Intercept)	58.00	9.95	5.87	**2.57e-06**
Diet: Control	-22.00	10.91	-2.02	0.0530
Diet: Vitamin E	-23.47	10.52	-2.23	**0.0336**
Diet: Wild	-0.50	12.19	-0.04	0.9676

3.5 | Discussion

The steady decline in ferret fertility may be linked to diet (Chapter Two). While diet has not been shown specifically to impact whelping rates or number of kits born, diet has been shown to significantly influence sperm DNA damage levels as well as to influence several other key sperm metrics that influence the ability for a dam to conceive (Chapter Two). Despite this fact, our RNA-Seq analysis finds that transcriptionally, individuals across the control, vitamin E-supplemented, carcass and wild diets do not vary drastically from one another. The unsupervised hierarchical clustering heatmap shows no evidence of clustering of individuals based on diet or wild vs. captive status.

3.5.1 |Transcriptomic trends in Wild ferrets

In wild ferret sperm, genes were upregulated in pathways related to microtubule movement compared to both vitamin E and control ferrets. This may imply that the wild diet somehow increases sperm motility through mechanisms related to dynein proteins, cytoskeleton and microtubule functioning. Additionally, the significance of olfactory pathways being enriched in mature wild ferret sperm is that olfaction in sperm is related to chemical detection of the oocyte as well as motility (Spehr et al., 2006). Generally, we observe higher sperm motility metrics in wild ferrets compared to control and vitamin E ferrets (though the difference is not

significant) (Chapter Two). Those eating a wild diet also had genes upregulated in metabolism and pathways related to bone development and telomere length compared to control ferrets. It may not be only the diet that influences these changes; wild ferrets live in their natural environments which are very different from the environments of captive ferrets. They must travel long distances each day to find and kill prey that are much larger than them, likely using their senses to a higher degree than captive ferrets, which are fed everyday by caretakers. Wild ferrets have been shown to have significantly larger ulna and tibia bones than *ex situ* ferrets; wild males had 9% larger limb length than *ex situ* males (Wisely et al., 2005).

Wild individuals, when compared with vitamin E individuals, had enhanced transcription in genes related to membrane rafts, which are present in the plasma membrane of sperm cells overlying the acrosome (Kawano et al., 2011). This is where fusion happens with the oocyte and is directly related to sperm capacitation, critical for fertilization (Priyadarshana et al., 2018). As previously mentioned, wild ferret acrosomal metrics were healthier than that of the other diets, and this may be related to the upregulation of genes related to the acrosome. Understanding the mechanism behind how the natural diet impacts transcripts related to sperm capacitation downstream needs to be investigated further.

3.5.2 | Transcriptomic trends in vitamin E and control ferrets

In control ferrets, we found that genes were upregulated in pathways related to microtubule movement and olfactory transduction compared to vitamin E ferrets. Some potassium channel related genes were also enhanced, which is critical for sperm capacitation, the process that allows sperm to penetrate the oocyte (Hirohashi & Yanagimachi, 2018). In wild ferrets, upregulation in microtubule movement and olfaction might imply that motility in sperm

is enhanced given that the wild diet is what ferrets evolved to eat, thereby providing the exact balance of nutrients they require. The assumption about the control diet is that it may be oxidative stress-inducing given the high levels of vitamin A in horsemeat and how excess vitamin A can antagonize vitamin E (Swick & Baumann, 1952). If vitamin E is antagonized, then there is not a proper balance of antioxidants to ROS and lipid peroxidation and other types of oxidative damage that can impact sperm cells (C. Wright et al., 2014). Perhaps control ferrets have pathways upregulated in genes that enhance microtubule movement to compensate for any oxidative damage that might have occurred to sperm cells during development. Alternatively, it could be that while the transcriptional rate for these genes is higher in control ferrets, the translational rate does not follow, i.e. there is not a higher number of translated proteins related to motility that actually increases motility metrics.

SPAM1 encodes an enzyme on sperm cells that is responsible for helping to penetrate through the zona pellucida of the oocyte, and it was significantly downregulated in vitamin E individuals compared to controls and enriched in cell adhesion functional cluster in controls compared to wild ferrets. Mice fed a high cholesterol diet also had reduced expression of *SPAM1* (Yang et al., 2020). A reduction of *SPAM1* mRNAs is associated with delayed fertility (Hermo et al., 2010), but we did not find this association to be true across any diets. *SPAM1* may be upregulated in control ferrets compared to wild ferrets because wild ferrets had significantly lower percent of abnormal acrosome and damaged apical ridge than control ferrets (Chapter Two), indicating that their acrosome health and integrity was superior to control ferrets. It is possible that control ferrets upregulated *SPAM1* in order to overcompensate for this decline in acrosomal health. Although vitamin E ferrets did not have a significantly lower percent of abnormal acrosome or damaged apical ridge than control ferrets, *SPAM1* was downregulated

which suggests that this transcript was not needed to improve chances of fertilization compared to control ferrets.

We found that G-protein coupled receptor (GPCR) signaling was an enhanced pathway in vitamin E individuals compared with control ferrets (Fig. 3.5A). GPCR and G-proteins are also associated with chemosensory detection, as is the olfactory transduction pathway that was enhanced in control ferrets. The enhancement of this pathway is more specific to olfactory detection and implies that vitamin E individuals have genes enhanced related to sperm detection of the oocyte. While we did not observe an improved fertility metric for vitamin E individuals, this finding may have downstream implications over several generations.

We also found that phagosome functions were enhanced in vitamin E individuals (Fig. 3.5B), which are critical for engulfing regressive tissues during spermatogenesis and thereby help to form functioning, healthy sperm (Tang et al., 2016). These transcripts can be left over from spermatogenesis, indicating an increased functioning during sperm development, potentially accelerating the production of spermatozoa (Shi et al., 2018). These genes are also associated with the inflammatory pathways and immune system functioning (Stuart & Ezekowitz, 2005).

In mature sperm, mRNAs can be critical for embryo development once inside the oocyte. A signal of oocyte activation is oscillation of calcium (Ca^{2+}), which is necessary for embryo development (Zafar et al., 2021). It has been shown that sperm mRNAs are critical for oocyte activation (Zafar et al., 2021). Ca^{2+} oscillations in the oocyte are controlled by phospholipase C zeta (PLCζ) by activating the Inositol 1,4,5-trisphosphate pathway (Zafar et al., 2021). While *PLCζ* gene was not differentially expressed in our study, several calcium-related genes, including *S100A8* and *S100A10*, were enriched in calcium ion binding and other functional terms

in vitamin E ferrets compared to controls. In wild compared to both control and vitamin E ferrets, *S100A10* was enriched in functional terms membrane raft assembly, membrane budding, and positive regulation of binding. *S100A10* has been reported to be present in the oviduct and related to early embryonic development processes (Teijeiro et al., 2016).

Control ferrets also had specific genes that were very highly expressed in comparison with wild ferrets. While many of them have not been described in sperm before, a few of them are well-characterized. For example, DNMT1 is critical for gene expression during spermatogenesis and infertile male patients having sperm maturation arrest lack the DNMTI protein in their seminiferous tubules (Omisanjo et al., 2007). DNMT1 also plays an important role in methylation patterns in DNA but a recent study could not find a correlation between DMNT1 transcripts and sperm DNA damage or sperm parameters (Rahiminia et al., 2018). Another study found that DMNT1 in mature bull sperm was significantly correlated with fertility (Menezes et al., 2020). The HSD11B1L protein catalyzes inactive forms of glucocorticoids to active forms, which are hormones that regulate the physiologic response to activation of the hypothalamus-pituitary-adrenal (HPA) axis (Liu et al., 2021). In a study in pig testis, HSD11B1L potentially played a role in preventing Leydig cell apoptosis and testosterone suppression caused by glucocorticoids (Ohno et al., 2013). The DEFB124 was associated with immune system defense in bovine epididymal sperm (Légaré et al., 2017). Perhaps control ferrets are upregulating DMNT1 compared to wild ferrets as a way to achieve improved fertility given some morphological or physiological limitations they may have due to the diet impact. And perhaps HSD11B1L and DEFB124 are upregulated as a way to control for immune response in the face of a diet that may be oxidative-stress inducing. The PRR14 has been shown to be the factor that tethers heterochromatin to the nuclear lamina in human HeLa cells (Poleshko et al., 2013).

Though no studies have shown this in sperm, perhaps PRR14 is upregulated in control ferrets in relation to chromatin integrity.

3.5.3 | Vitamin E supplementation, DNA damage and oxidative stress

In Chapter Two, we determined that second-generation vitamin E individuals had lower levels of DNA damage in mature sperm than first-generation vitamin E ferrets. Lower levels of DNA damage in second-generation vitamin E mature ferret sperm might imply that some gene pathways are activated that help repair the mechanisms that lead to DNA damage. Because we found that blood serum total antioxidant capacity (TAC) and sperm DNA damage levels were lower in second-generation vitamin E ferrets, we hypothesized that transcripts related to the antioxidant defense system might be upregulated, helping to ward off oxidative stress and thereby lower DNA damage levels. However, we did not find functional terms related to the antioxidant defense system pathways enriched in any specific diet group. This may imply that the mechanism leading to the higher levels of DNA intactness in second-generation vitamin E ferrets is not necessarily related to reducing oxidative stress on a transcript level in mature sperm.

The hypothesis from Chapter Two related to sperm DNA damage in ferrets is that their DNA became vulnerable to damage due to aberrant protamine ratios caused by inbreeding depression. Inbreeding depression was hypothesized to have revealed an allele that may have altered the functioning of protamine genes (P1 and P2; Balhorn, 2007) across the entire ferret population, regardless of diet and captive or wild status, since all ferrets stem from the same gene-pool. Protamines are sperm-specific proteins that replace histones and condense DNA ten times as tightly (D. Miller et al., 2010). This extra condensation increases protection of DNA

from nucleases and oxidizers in the female reproductive tract (Gunes et al., 2015; Rathke et al., 2014; Tarozzi et al., 2009). When protamine ratios or the transition process is compromised, DNA can become vulnerable to damage (Oliva, 2006). Protamines are critical for normal fertility – aberrant ratios lead to high levels of DNA damage and sterility (Miller et al., 2010). We hypothesize that the captive diet is causing higher levels of oxidative stress (due to excess vitamin A), which thereby may increase ROS attacks on the DNA made vulnerable by the aberrant protamine ratios, whereas the wild diet does not promote oxidative attacks on the DNA even though it is just as vulnerable.

We expected to find transcripts upregulated in vitamin E ferrets related to protamine transcription, storage or translation, since their levels of DNA damage were lower. Perhaps the protamine protein ratios were restored in second-generation vitamin E ferrets, implying less vulnerable DNA and thereby, lower DNA damage levels. However, we were unable to detect differential expression in the genes cited in studies on protamines (Carrell et al., 2007) in mature sperm across diets. This might imply that protamine protein ratios were intact across all diets and that DNA damage may have occurred in sperm before spermiogenesis, when the histone-protamine transition takes place. When sperm cells still contain histones, they are more vulnerable to DNA damage than they would be with protamines, and it is possible that the damage we observe occurs via oxidative stress at this earlier stage during sperm development.

Our study did find an overlap with a 2015 study on human mature sperm that looked at differentially expressed genes in fertile vs. infertile individuals (Bansal et al., 2015). These authors found that SMARCAD1, a transcript responsible for a protein that is involved with chromatin repair and organization, was downregulated in infertile, asthenozoospermic individuals compared to fertile, control individuals. This implies that there may be less DNA

repair mechanisms in infertile individuals compared to fertile ones. In our study, SMARCAD1 was enriched in protein homooligomerization function (potassium channel cluster) in vitamin E diet group compared to control (Fig. 3.5A). Both first and second-generation vitamin E ferrets were combined in our results evaluation, but 82% of these were second generation vitamin E. The observed enrichment of the SMARCAD1 transcript may be playing a role in the reduction of DNA damage occurring in second-generation vitamin E ferrets.

If DNA damage occurred during sperm development, SMARCAD1 may have been upregulated during that stage in response to it before the protamine transition, and the transcripts we observe may be residuals from that process. But, it is also possible that DNA damage occurred once the protamine transition took place and that SMARCAD1 was upregulated in response to that, once sperm is technically regarded as transcriptionally inactive. SMARCAD1 may have been able to be transcribed during this time period because sperm retain some transcriptional and translational activity after maturation (Carrell et al., 2007; Gur et al., 2006), which may in part be due to the fact that sperm retain anywhere from 1 to 15% of histones; in humans it's been detected as 4% (Hammoud et al., 2009; M. et al., 1987). Studies have shown that these retained histones actually contribute to the zygote genome and are enriched at promoters related to development of the embryo (Hammoud et al., 2009; van der Heijden et al., 2008).

There is evidence that machinery still exists in mature sperm to transcribe histones (Ren et al., 2017). The *SMARCAD1* transcripts we observe may have been transcribed via genes in retained histones in response to DNA damage that occurred post-protamine transition. This has been shown in other studies as well. For example, *SMARCAD1* is upregulated in cryopreserved human sperm, which suffers from DNA damage due to the freeze-thaw cycle. This indicates that

this repair mechanism is activated to compensate for increased DNA damage which takes place in mature sperm, after most transcription stops taking place (Shangguan et al., 2020). Similarly, in giant pandas, cryopreserved mature sperm was upregulated in lncRNAs and mRNAs related to the cold response and apoptosis compared with fresh sperm (Ran et al., 2018).

Our lack of evidence related to transcripts in oxidative damage control may also imply that DNA damage is lower in second-generation vitamin E ferrets not based on transcriptional differences but completely unrelated to gene expression altogether. It is known that seminal plasma helps to protect sperm DNA since it is rich in antioxidant enzymes (Khosrowbeygi & Zarghami, 2007). Sperm cells are most vulnerable to oxidative damage after spermiogenesis, when they are stored in the epididymis for 12-14 days, without the additional of seminal plasma (Bisht et al., 2017). In our previous study we investigated whether TAC differed across seminal fluid and did not find evidence that it was different in second-generation vitamin E individuals. However, we did find a significant negative correlation between DNA damage and seminal fluid TAC - low levels of TAC were associated with higher levels of DNA damage. This may indicate that the higher the ability of seminal fluid to protect against ROS, the less DNA damage will occur. These results combined with the fact that we did not observe any enrichment in oxidative stress pathways may suggest that the lower DNA damage observed in second-generation vitamin E ferrets is because antioxidant levels in serum and seminal fluid help protect the vulnerable DNA from damage.

3.5.4 | Impact of vitamin E supplementation

Genes that were upregulated in the vitamin E supplemented diet were compared with other studies that investigated at the effects of vitamin E supplementation. A study on sperm taken from sheep testes found that vitamin E supplementation enhanced transcription in genes related to antioxidant activity and improved reproduction for the animals overall (Xu et al., 2016). Researchers found increased enzyme activity related to glutathione, implying that the increased amount of antioxidant availability allowed for an increase in using antioxidants for various functions (Xu et al., 2016). The main genes related to these functions, *GSTA1* and *GPx3*, differed across various levels of vitamin E supplementation in sheep testes. For example, supplementation with 2000 IU of vitamin E did not differ in terms of *GSTA1* expression level compared with no-supplementation, 20 or 100 IU of vitamin E, but 200 IU had significantly higher expression levels than no-supplementation. Ferrets were supplemented with 300 IU of vitamin E but there was no differential expression of these genes detected. It is possible that the high levels of vitamin A present in horsemeat suppress the effects of vitamin E, which subsequently impacts the lack of glutathione activity we observe. Additionally, this differential expression was observed in sperm from sheep testes (Xu et al., 2016) and it is possible that such differences are not detectable in mature sperm.

We did not observe an improved reproductive outcome in our vitamin-E supplemented individuals compared to controls, as was found in the sheep testes study (Xu et al., 2016); however, one gene related to antioxidant activity was upregulated in control compared to vitamin E diet group. Jervis and Robaire (2004)found that vitamin E deficiency in rats observed increased expression of superoxide dismutase 1 (*Cu-Zn SOD1*) in the corpus epididymis. The sheep testes study found similar genes enhanced in vitamin E deficient individuals (Xu et al.,

2016). In our study, *SOD3* was enriched in functional terms related to cytoplasm and plasma membrane in control compared to vitamin E group. These *SOD* genes encode superoxide dismutase proteins, which are responsible for converting superoxide radicals into hydrogen peroxide and oxygen, thereby neutralizing potential oxidation events. This result may imply that the control diet is upregulating these mechanisms compared to the vitamin E diet, which may provide more antioxidant activity via vitamin E intake. On the other hand, compared to control ferrets, the vitamin E ferrets in our study showed upregulation in genes such as *PPM1A*, which can inhibit activation of cell stress regulators (Lammers & Lavi, 2007). This finding may imply that the vitamin E diet resulted in less activation of these cellular stress response pathways since the vitamin E supplementation enhanced antioxidant activity. Alternatively, *PPM1A* is also involved in oocyte maturation (Chuderland et al., 2012) and was enhanced in nucleus and membrane functional terms, so may in fact be unrelated to the stress response in this context.

3.5.5 | Study limitations and next steps

Only 6 of the 33 ferrets in this study were evaluated for their vitamin serum levels to validate the increase of serum vitamin E levels during supplementation. However, a previous study found that serum vitamin E levels were not significantly different between control or vitamin E-supplemented individuals (either across first- or second-generation vitamin E supplemented) (Chapter Two). However, that study did find that TAC serum levels, which are a proxy for antioxidant levels, were significantly lower in second-generation vitamin E individuals. This may indicate that vitamin E supplementation has transgenerational effects which result in less activation of the antioxidant pathways that ward off oxidative stress. Alternatively, being fed vitamin E supplementation in early development may result in less need

for activation of the antioxidant pathways later in life. With either theory, we have some validation that vitamin E supplementation had an impact on the physiological response of ferrets. However, based on our results from the PCA and hierarchical clustering, the differences between different diets groups are not large enough to detect major differential expression in these areas of interest (Fig. 1).

It is also worth noting that transcripts retained in the cytoplasm are a small portion of what was transcribed before the cytoplasmic droplet falls off during spermatogenesis (Rengan et al., 2012). The transcripts that are left in mature sperm are a fraction of what was present during spermatogenesis (Jodar et al., 2015), and therefore, the fraction that we measure may not be providing a full or true picture of what is happening in the transcriptome. Further, very little is known about ferret spermatogenesis, histone retention, and response mechanisms. It is possible that oxidative damage took place to sperm cells early on during development but that early stage cells are incapable of mounting a response, which is why we do not observe certain pathways enriched.

Further, seminal plasma contains important transcripts that play a role in female ovulation as well as increase the expression of inflammatory response pathways in endometrial tissue in the female reproductive tract (Bai et al., 2018; Jodar, 2019). Our study focused only on sperm cell RNAs, so it is possible that differences in transcripts due to diet treatments were present in the seminal plasma that we did not analyze. Future studies should retain seminal plasma separately from sperm cells to determine its unique transcriptomic profile and whether differential expression across treatment groups exists.

Lastly, because mature sperm is mostly transcriptionally inactive, the presence of transcripts does not prove that these proteins are necessarily being translated at whatever

quantity they appear to be present (Darszon et al., 2011), although proteins related to translational regulation are found in sperm cells (Amaral et al., 2014). While we observe many pathways in wild ferrets enriched in genes that may be related to sperm morphology such as motility and acrosome health, we observe pathways in control ferrets enriched in similar pathways compared to vitamin E ferrets. Control ferrets do not have increased health in these sperm parameters. Therefore, we cannot make claims that these transcripts are directly related to the evidence of increased sperm health in wild ferrets. Proteomic analysis would help us assess whether the transcripts present in mature sperm actively translate to similar quantities as proteins, which would help validate some of the claims we have made here.

3.5.6 | Summary

In summary, we find enhanced functions in wild individuals in genes related to sperm cellular movement and chemosensory detection in mature sperm. Functions enhanced between the control and vitamin E diets are not as differentiated compared with the wild diet; we observe some overlaps in upregulated functions in both diets related to olfaction, GPCR signaling, and potassium channels that are critical to the acrosome reaction. We do find some increased activity in microtubule movement related to control ferrets.

While we did not observe large swaths of genes related to antioxidant activity upregulated in response to the vitamin E supplementation, we did find evidence of one gene involved in antioxidant repair (SOD3) that might help regulate oxidative damage in control ferrets. We also found a transcript involved in chromatin repair (SMARCAD1) enriched in the protein homooligomerization functional pathway in vitamin E ferrets which may be related to improved DNA damage levels. Additionally, finding SPAM1, involved in sperm capacitation,

upregulated in control ferrets may indicate a compensation mechanism to improve the capability of sperm to penetrate the oocyte, although there is no evidence of superior reproductive capabilities in these ferrets.

Overall, our work is one of a limited number of studies that examines sperm cell transcriptomics in a non-model organism and in an endangered species. The transcriptomic analysis we have conducted has only been done in two other non-model, wildlife species, the Giant Panda (*Ailuropoda melanoleuca;* Ran et al., 2018) and the European rabbit (*Oryctolagus cuniculus;* Schuster et al., 2016). Our work investigates the possibility of EDID through transcriptomic evaluation of genes under differing environmental circumstances. This work serves as a model for studies in other endangered or threatened species that may be experiencing inbreeding depression compounding with environmental factors that impact reproduction.

CHAPTER FOUR | Machine learning as a tool to predict reproduction outcomes and determine factors that influence reproduction in an endangered carnivore population

4.1 | Abstract

Reproduction is often intensively managed in managed, *ex situ* (captive) populations of endangered species, and is a vital component of conservation. However, predicting whether a particular pairing will be successful is difficult and what makes a reproductive event successful is often chance. However, years of high-quality data are collected by managers and researchers and can be used to analyze what influences reproduction and what potential adjustments can be made for increasing future success. Our aim was to: 1) determine if machine learning (ML) could be used to predict reproductive outcomes and 2) determine what factors influenced the reproductive outcome using data from almost 20 years of the black-footed ferret (*Mustela nigripes*) recovery program. Factors assessed included biographical information such as age, kinship, and past experience, and physiological data such as ejaculate quality. Using ML, we derived a random forest model that accurately predicted reproductive outcome 82% of the time using spermatozoa characteristics from artificial insemination events. Models using different factors also improved on a baseline model, but not to the same degree of accuracy. Our statistical models indicated that factors such as past successful reproductive outcomes, cumulative reproductive experience, and acrosomal integrity of spermatozoa significantly influenced reproductive outcome. These results suggest ML can be used to inform the management of captive species and that reproduction in carnivores is influenced by many factors, some of which can be improved upon by management and scientific intervention.

4.2 | Introduction

The Global Assessment Report on Biodiversity and Ecosystem Services (IPBES) detailed evidence that one million species, both plant and animal, were at risk of extinction (Brondizio et al., 2019). The leading cause is changes in land and sea for human use. This loss of habitats has necessitated *ex-situ* management of many species in zoos and aquaria (IUCN SSC, 2014). However, captive management poses many of its own challenges, including small population sizes and low genetic diversity, leading to unsustainable long-term biological viability (Che-Castaldo et al., 2019). The two major factors that contribute to unsustainable populations were space limitation and insufficient reproduction (Che-Castaldo et al., 2019). Many factors can limit breeding success, including individual breeding history, fecundity, mate choice, space, and other management considerations (Bauman et al., 2019; Che-Castaldo et al., 2019). To understand which factors influence species fecundity and population growth, data often spanning many years and stemming from multiple sources, including studbooks, breeding facilities, researchers, and managers, must be collated and analyzed.

In addition to understanding the drivers that affect reproductive success, population managers want to predict whether a particular pairing will be successful. Currently, the ability to make these predictions is poor. Managers rely on population management software and pedigree analysis to determine ideal pairings, but they also must rely on historical and anecdotal information about individuals and pairs, such as behavior, temperament, and past mating events, to determine whether they will be a productive match (Asa et al., 2011). However, the complexity of understanding what factors contribute to whether a pair will mate and whether the female will come to a full-term pregnancy often leads managers to rely on already proven breeders (those that have successfully produced offspring before), but those individuals are

usually thereby less genetically valuable for the breeding program (Asa et al., 2011). Being able to predict the success of a pairing allows us to optimize which individuals to pair together and can lead to better genetic management of the population overall. Additionally, this increases the number of ferrets reproducing in the managed population, thereby increasing the effective population size.

A potential approach to forecast breeding success is through predictive modeling. For example, machine learning (ML) has recently been employed for this purpose in conservation (Kwok, 2019; Wearn et al., 2019). ML refers to any collection of tools that is aimed at making accurate predictions from a given set of data (Lucas, 2020). ML models can analyze nonparametric data and nonlinear relationships and incorporate interacting covariates to arrive at predictions without a-priori knowledge or hypotheses. Specifically, the algorithms generate highly complex relationships between the independent and dependent variables, often approximated by nonlinear and high dimensional relationships with interacting factors (Zhao & Hastie, 2021). In conservation work, where data are often deficient, sparse, and non-normal, traditional statistical approaches will lack predictive power. ML, however, is robust to such issues and has proven useful for making predictions in data deficient species (Bland et al., 2015).

In this study we use the endangered black-footed ferret (*Mustela nigripes;* ferret) as a case study to test the abilities of ML to predict whether a particular pairing will be successful and determine which factors affect success. Once extinct in the wild, the ferret is now bred in human care and managed across six North American facilities (Graves et al., 2019). A studbook, a comprehensive database of individual-based parentage and life history, has been maintained for the past 30 years of *ex situ* management. Additionally, physiology data including seminal traits

are collected. These data could be used to understand why and how reproductive success in this species is on the decline (R. Santymire et al., 2018).

Inbreeding, the excess probability beyond chance of two alleles being identical at a given locus in an individual (Charlesworth et al., 1987), likely explains in part a steady decline in whelping rates (60% in the 1990's to 46% in 2021), since the ferret population was initiated with only seven individual founders (Miller et al., 1996). Species with a small founder population are at a higher risk for inbreeding depression which may be characterized by issues like sperm degradation (Brekke et al., 2010; Fitzpatrick et al., 2009) and reduced pregnancy rates (Keller & Waller, 2002). Inbreeding depression can negatively impact sperm quality, including motility, concentration, and morphology (Fitzpatrick et al., 2009; Malo et al., 2010). Santymire et al. (2018) recently established that increasing inbreeding (*F*) over time was significantly correlated with declining normal sperm traits in ferrets. Normal sperm morphology has declined from 50% in 1990 to 35% in 2021 (R M Santymire et al., 2014, 2006). As there is a strong correlation between low fertility and highly abnormal ejaculates, this decline poses a critical threat to the future of the species (Pukazhenthi et al., 2006).

Our goal was to dig deeper into understanding what factors influence reproductive outcome when taking into consideration all pairings from all facilities since 2004. Our aim was two-fold, and we therefore followed two approaches to help us understand the influences on reproduction. Our first aim was to test whether various factors could be used to predict reproductive outcome for any given pair. For this aim, we followed an ML approach. We hypothesized that given the rich datasets we have for the ferret, ML models would allow us to predict to a higher degree of accuracy than a baseline model, reproductive outcomes for a given mated pair. Kinship of the parents (*F* of the would-be litter per pair) could be a factor driving

reproductive success, with more closely related individuals (the ones that would produce offspring at higher levels of inbreeding) having lower reproductive success. Additionally, given past research on the importance of sperm characteristics (Santymire et al., 2018), we hypothesized that seminal traits would also be highly predictive of reproductive outcome. Lastly, we hypothesized that an individual's past successful reproductive outcomes would make them more likely to have positive future reproductive outcomes.

Our second aim was to understand better the underlying relationships driving reproductive success. For this aim, we followed a statistical modeling approach. Statistical modeling or inference is more traditionally pursued in biology and ecology and focuses on parametric modeling, with pre-defined relationships amongst parameters underpinned by a likelihood function that results in a probabilistic model (Bzdok et al., 2018; Lucas, 2020). While ML may provide predictions about outcomes, having a way to relate these models to existing literature and our understanding of the ferret's biology is crucial. Here, we hypothesized finding significant influences on reproductive outcomes from the parameters mentioned above – kinship, seminal traits, and past reproductive outcomes.

4.3 | MATERIALS AND METHODS

4.3.1 | Study species

4.3.1a | Animal research

This research was reviewed and approved by Lincoln Park Zoo's Research Committee (proposal #2007–005) and United States Fish and Wildlife Service (Carr, CO). No animals were euthanized during this study. All animal studies conformed to the Guide for Care and Use of Laboratory Animals.

4.3.1b | Breeding Facilities

Reproductive data were collected by the six ferret breeding centers across the Species Survival Plan® (SSP) of the Association of Zoos and Aquariums (AZA). Breeding facilities include the USFWS's National Black-footed Ferret Breeding Center (FCC); National Zoo's Smithsonian Conservation Biology Institute (SCBI); Louisville Zoological Gardens (LZG); Cheyenne Mountain Zoo (CMZ); Phoenix Zoo (PHX); and Toronto Zoo (TOR). While each facility collects relatively similar data, data types were not uniform. As such, individual breeding reports from each year available per facility were combined and standardized in Python (Version 3, Van Rossum & Drake, 2009). Breeding records spanned 2004 until 2020, but not all facilities have data records for each year (Table 4.1). When necessary, managers were contacted to understand context related to notes taken on specific individuals or to understand how certain metrics were determined. Records were excluded when they did not match strict criteria of having traceable and updated metrics from the studbook including date of birth, death, lineage information, inbreeding coefficient (*F*; metric associated with level of inbreeding depression; see Table 4.1), or facility.

Table 4.1. Definitions of the predictors included across our four datasets, taken from data spanning 2004 to 2020 from the six black-footed ferret breeding facilities

Variable	**Definition**
Age difference	Difference in ages between a male and female (older individual's age minus younger individual's age).
Breeding year	Year that breeding took place
Dam & Sire age	Age of the individual at the time of breeding
Dam & Sire birth year	Year an individual was born.

Variable	Definition
Dam & Sire cumulative count	How many times an individual had a breeding attempt prior to the current breeding attempt. If an individual had more than one breeding attempt in a year, the experiences were labeled in an arbitrary order.
Dam & Sire F	The inbreeding coefficient for each of the prospective mother and father. Inbreeding coefficient measures the probability that the two alleles at a genetic locus are identical by descent (S. Wright, 1922).
Dam & Sire IDs	IDs are taken from the studbook to uniquely identify individuals.
Facility	The facility in which a ferret resided at the time of the breeding event.
Kinship	The probability that a random allele from one individual is identical by descent to an allele chosen at random from that locus in the other individual (Lacy, 1995).
Mating type	Whether a breeding attempt was natural (natural pairing event) or through artificial insemination (AI)
Outcome 1, 2 & 3	A binary outcome (whelped or did not whelp) resulting from a breeding attempt. Outcome 1 and 2 are predictors; outcome 3 is the dependent variable in our models.
Sperm % motility	Percent of sperm from a sample of 100 sperm that are fully motile.
Sperm motility status	Average forward progression of the motile sperm in the sample (1-5, 1 being slow, 5 being fastest and straightest).
Sperm % normal acrosome	Percent of sperm from a sample of 100 sperm that have a fully intact and normal appearing acrosome.
Year of Artificial Insemination	Year in which the artificial insemination took place.

4.3.1c | Reproductive Physiology

Managers from each facility monitor males and females leading up to the breeding season (February through June). Vaginal epithelial cells become cornified as estrogen levels increase (E.

S. Williams et al., 1992). When the percentage of cornified epithelial cells exceeds 90%, females are considered in estrus and are paired with a male 5-7 days later. Following pairing, a vaginal lavage is conducted to check for the presence of spermatozoa (sperm check) (Williams et al., 1992).

For males, testes firmness is an indicator of sperm production and breeding readiness (E. S. Williams et al., 1991). We have seminal data for a portion of the males in our study. Semen was collected from these anesthetized males by electroejaculation (Howard, 1993). Researchers evaluated sperm for percent motility and forward progression (0-5; 5 is the highest score); sperm morphology, including acrosomal integrity, was assessed using phase-contrast microscopy (1000×) on 100 sperm cells per sample (Santymire et al., 2006).

While most individuals were bred naturally (paired in enclosures), a subset of individuals had artificial pairings via artificial insemination (AI) using an inter-uterine laparoscopy method. Data on seminal quality was compiled from AI events spanning 2008 to 2015. Both fresh and frozen semen was used for AI depending on the individual and the year. Cryopreservation and AI protocols are detailed in Howard et al. (1993).

4.3.1d | Pairing

Managers pair individuals based on genetic breeding recommendations from the population management advisor (C. Lynch, Riverbanks Zoo, Columbia, SC) utilizing methods typical of managed populations (Ballou et al., 2010), as well as on historical and anecdotal data. Breeding and reproduction data is subsequently collected, including date of pairing, sperm check, whelp date, and notes on irregularities or behaviors, such as kit cannibalism (Table 4.1).

4.3.1e | Reproductive data

Relevant data from the studbook as well as reproductive data from each facility were combined by matching on pairs specific to that year (Table 4.1). During data cleaning and compilation, all breeding records were assigned a breeding "outcome". Breeding outcomes fell into one of four categories: "whelped", "did not whelp" ("DNW"), "did not ovulate" ("DNO"), and "other". Ferrets are induced ovulators (Chang, 1965); if a female does not ovulate after mating, she is unable to become pregnant. Sometimes females that did not ovulate after natural breeding were paired with another male or were given porcine luteinizing hormone (Sioux Biochemical) to stimulate ovulation. If she did not ovulate, she was placed into the "DNO" category. Females that did not come into estrus were also placed into this category. Breeding records that were placed into the "other" category resulted in an incomplete mating or gestation for various reasons, including but not limited to: female death after mating, incompatible pairing, illness, and surgical or health procedures. The "whelped" category was used for any female that gave birth, even if her kits were stillborn or cannibalized. The "DNW" category was used for individuals that ovulated, were mated and had a positive sperm check but did not produce offspring.

Our final dataset was filtered only on the binary outcomes "whelped" and "DNW", as we were interested solely in predicting whether a pair had kits or did not have kits. Focusing on whether a successful pairing resulted in a full-term pregnancy or not allows us more power to predict the outcome using parameters of interest.

4.3.2 | Data analysis

We conducted two separate analyses, ML and statistical analyses, on the same four datasets (described below). These datasets used different but sometimes overlapping sets of predictors and data.

Dataset A had the most data at 1890 records. The variables used in each model are summarized in Table 4.2 and 4.3. For Dataset A, each record included breeding data between one male and one female. Variables included sire and dam studbook numbers (IDs), breeding year, facility, breeding outcome, and dam and sire age at time of reproductive event (Paul Marinari, 2020). Dam and sire *F*, as well as kinship, were generated from studbook data in PMx (Lacy et al., 2012). Any individuals that did not have an *F* metric (usually due to an unknown parent) or a kinship to their mate were removed from the analyses. Additionally, cumulative count was calculated for each individual in a pair. This metric determined how many times the individual had a breeding attempt prior to the current breeding attempt. It was used as a proxy for how experienced a breeder an individual was. If an individual had a breeding attempt more than once in a year, the experiences were labeled in an arbitrary order.

Dataset B is a subset of Dataset A that contained male spermatozoa characteristics for that specific breeding year's AI events. It contains 83 records. To assess whether semen parameters impacts breeding outcome, the quality for the specific ejaculate deposited into a female must be known, since seminal quality changes from ejaculate to ejaculate (Vega-Trejo et al., 2019).

Dataset C explored the predictability of past breeding outcomes. For example, if a male was a first-time successful breeder, would he be a successful breeder the second time? It contained 272 records. Dataset A was filtered to males that had three or more breeding records.

Then, the first three sequential breeding outcomes associated with a male were mapped back to him. Because each male now had three outcomes associated with him rather than just one, all data associated with individual breeding events had to be removed (i.e., female ID, female F, breeding year, etc.). If a male was transferred to another facility during his breeding lifetime, the facility where he was born was used as his designated facility. The predictors in this dataset were focused on male attributes and past outcomes (Table 4.2). This dataset tests if male factors are largely responsible for breeding outcomes since the breeding outcomes are not paired with dam-identifying information.

Dataset D tests if female factors are largely responsible for breeding outcomes, regardless of the male to which she is mated. It contains 385 records. The same process was followed as for Dataset C, except focused on female breeding records.

The variables in each dataset were categorized differently based on which analysis was run (Table 4.2 & 4.3). For the ML models, years and ages were converted to factor variables to consider each individual year or age as a contributor to reproductive outcome. In our statistical models, especially for the small datasets, the models would not converge if there were too many parameters. Converting ages and years to factor overcomplicates the statistical model as it adds several more parameters to the list of predictors, which will cause overfitting or non-convergence. Therefore, ages and years were included as continuous variables in the statistical models.

Table 4.2. Classification of variables used in our machine learning models to predict reproductive outcome in the black-footed ferret.

Dataset	Numerical variables	Factor variables
A	Sire F, Dam F, Kinship, Sire cumulative breeding count, Dam cumulative breeding count	Sire ID, Dam ID, Breeding year, Sire age, Dam age, Sire birth year, Dam birth year, Age difference, Facility, Mating type
B	Sperm % motility, Sperm motility status, Sperm % normal acrosome, Dam F, Sire F, Kinship	Sire ID, Dam ID, Year of AI, Dam age, Sire age
C	Sire F	Sire ID, Breeding year, Sire birth year, Facility, Outcome 1, Outcome 2
D	Dam F	Dam ID, Breeding year, Dam birth year, Facility, Outcome 1, Outcome 2

Table 4.3. Classification of variables used in our statistical models to determine significant factors that influence reproductive outcome in the black-footed ferret.

Dataset	Numerical variables	Factor variables	Random effect
A	Sire F, Dam F, Kinship, Sire cumulative breeding count, Dam cumulative breeding count, Breeding year, Sire age, Dam age	Sire ID, Dam ID, Facility	Sire ID, Dam ID
B	Sperm % motility, Sperm motility status, Sperm % normal acrosome, Dam F, Sire F, Year of AI, Dam age, Sire age	Sire ID	Sire ID
C	Sire F, Sire birth year	Facility, Outcome 1, Outcome 2	
D	Dam F, Dam birthyear	Facility, Outcome 1, Outcome 2	

4.3.3 | ML Approach

4.3.3a | Pipeline

Supervised ML algorithms were used to predict binary reproductive outcomes given specific variables as predictors. At the start of the ML pipeline, the datasets are randomly split into a *training set, test set and validate set* (Fig. 4.1). The *training set* contains a portion of the dataset to train the algorithm. The *validation set* reserves a portion of the dataset for the algorithm to practice what it has learned from the training set. For example, it tests what it has learned by making predictions about the dependent variable in the *validation set*. The accuracy of those predictions is reported using a model score, which presents the accuracy of the predictions compared to the true values. These accuracy scores are used to select tuning parameters for the models.

The training and validation sets are used to maximize the accuracy of predictions while also not overfitting to the training set. If the algorithm is too well fit to the training set, it will not be able to predict outcomes for new data. By tweaking algorithm hyperparameters during the train-validation stage, which control various aspects of the learning process, one can maximize validation scores as well as test scores.

The *test set* is used as the final assessment of the model. The algorithm is fed a portion of these data that has been unknown until now, and it makes predictions based on this new data. How well the model does in the test phase explains whether the model overfit on the training set or whether it was robust enough to accurately predict outcomes based on new data.

The test set reserves 20% of the dataset for this final assessment. This leaves 80% of the dataset for the training and validation sets. A technique known as K-Fold cross-validation is employed to randomly split the training and validation sets. K-Fold cross-validation splits the

remaining 80% of these data into k parts randomly (ten parts is often used) (Hastie et al., 2001). Nine of these parts are used for training and one part is used for validation. The model is trained, validated, and then evaluated using an accuracy score metric. The mean of the ten scores helps evaluate how well the algorithm performed before it performs on the test set. If the mean score shows relatively low accuracy, hyperparameters must be tweaked or a new model should be built altogether.

The final test dataset should only be used to discern between models that performed the best. If several different algorithms performed well on train-validate sets, running test sets for each can help identify which algorithm is best for the dataset.

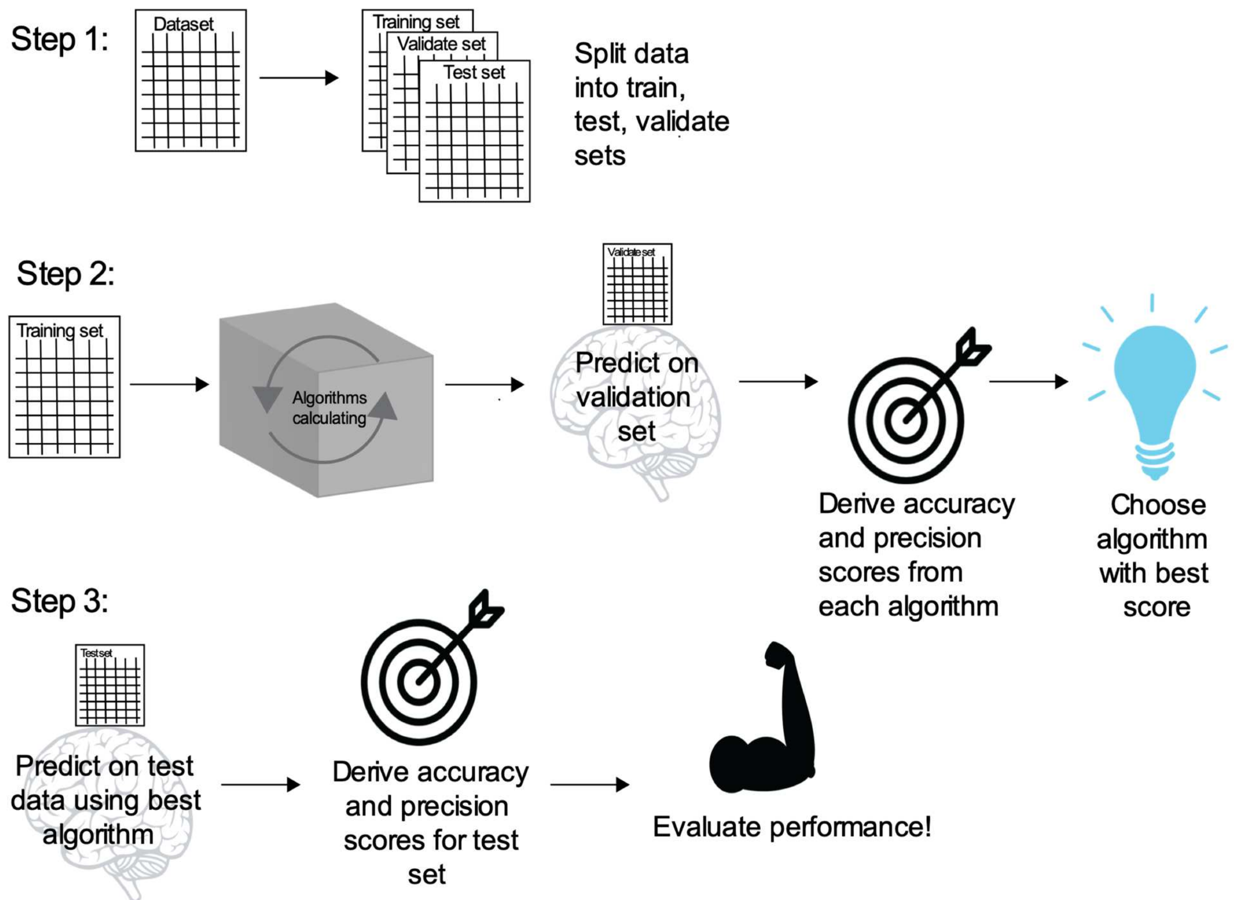

Figure 4.1. General steps in a machine learning pipeline. In step 1, the original dataset is randomly split into train, test, and validate sets. In step 2, the training set is used to train the algorithm. Then, the trained algorithm uses the factors of interest in the validation set to make predictions about the dependent variable. Accuracy and precision scores are generated by comparing the predictions to the true values. The best scoring algorithm is chosen to run the test set. In step 3, predictions are made using the test set, and model scores are generated as before.

4.3.3b | Threshold setting

In binary classification problems, the algorithm predicts the outcome as either 0 or 1 (in our case, DNW or whelped). Probability thresholds help guide this prediction. The default threshold is 0.5, i.e., where any outcome whose probability is greater than 0.5 will result in a classification of 1, and any outcome whose probability is less than 0.5 will result in a classification of 0. The default threshold of 0.5 can be adjusted for specific datasets using

threshold moving. Therefore, we tuned the threshold for each dataset since imbalanced datasets like ours can suffer from poor performance scores using the default 0.5 threshold (Provost, 2000).

For each model, a custom threshold was derived during the train-validation step. Predicted outcomes were reported as probabilities from 0 to 1 on the validation set. Then, we evaluated these probabilities using the ROC curve metric, which takes the predicted value and the true value as arguments and determines true positive rate, false positive rate, as well as ideal thresholds. A threshold was derived for each k-fold with the highest accuracy, and the mean of all the thresholds that led to the best classification accuracy was used as the final custom threshold for the model.

The custom threshold was then used to predict classification accuracy on the training set combined with the validation set (training_validation set), which serves as a final step before trying the model on the test set. The default threshold was also used to predict classification accuracy on the training_validation set as a comparison. Whichever threshold performed better was then used as the threshold for the test set.

4.3.3c | Algorithms

There are several types of ML algorithms for binary classification data. We tested logistic regression and random forest (RF) models. Logistic regression models classify data based on penalized likelihood predictions by seeking to minimize the cost function, or the logistic function (Lever et al., 2016). RF are ensembles of decision trees. Decision trees are built on recursive partitioning, where these data are split into partitions at nodes based on certain variables until it reaches a conclusion about the outcome. Each tree is trained on a random sampling (with

replacement) of data points and splits nodes based on a subset of the features which are selected randomly. The outcome is the average of all the trees (Hastie et al., 2001).

4.3.3d | Model Scores

Accuracy is an important model evaluator, defined as the percent of accurate predictions made. However, in a classification problem, if there exists a bias towards one class, accuracy may not properly evaluate the robustness of the model. For example, if 90% of the outcomes were “whelped” and a basic, baseline model predicted one class (or the other) 100% of the time, the accuracy score may end up being 90% if the baseline model predicts the majority class for all outcomes. This would imply the model is highly accurate when really it has just predicted the majority class for all outcomes, which requires no learning. Therefore, a baseline accuracy score was calculated for each model where the majority class was predicted for all outcomes. The accuracy score was then compared to this baseline score. If it was higher than the baseline score, then it can be assumed that the model has improved its guesses by learning.

Precision is the number of accurately predicted positive outcomes (whelped) over the total number of predicted positives (TP/(TP+FP)). It tests the ability of the model not to label a case of DNW as whelped.

4.3.4 | Statistical analysis approach

While ML methods are effective at making accurate predictions, if we want to interpret model fits and have valid uncertainty estimates for our parameters, a statistical framework is more appropriate. Additionally, statistical methods allow us to incorporate random effects into models. This means that the random variability from measuring traits across individuals can be

incorporated into the model. This improves our ability to make inferences about how fixed effects influence outcomes and helps to explain variance in our model. Only recently has the ability to add random effects to ML models been achieved (Hajjem et al., 2014).

There are multiple model selection methodologies that could be used. One of the most commonly used in ecology and conservation work is the stepwise multiple regression, which selects estimators that influence the dependent variable and tunes the model using forward or backward elimination based on changing significances (Whittingham et al., 2006). While this strategy is widespread, it has many limitations. Parameter estimation can be biased due to the fact that we adjust the model to our data, which increases the likelihood of overestimation of effect sizes (Hegyi & Garamszegi, 2011). This type of bias happens when parameters are added or excluded to a model and not compared to a suite of other possible models (Whittingham et al., 2006). Additionally, the direction of elimination or addition of parameters to the model has an impact on significance and scores, and therefore ultimate model selection. P-values are also not necessarily reliable indicators of significance, since parameters that fall above the threshold will be thrown out, when differences in p-values have been shown to be influenced by unpredictable factors (Thiese et al., 2016). Lastly, a "best" model may be selected when other, similar models may have an equivalent fit, leading to incorrect conclusions about the biological system (Whittingham et al., 2006).

Informational theoretic (IT) approaches have been proposed as robust alternatives to stepwise regressions. In an IT approach, an average model is constructed, where relative support for each parameter is deduced based on all informative models. Model importance can be deduced using Akaike's information criterion (AIC) (Hegyi et al., 2011). This precludes the use of p-values, though p-values are still provided in the model summary.

We followed an IT approach using the dredge function in the *MuMIn* package in R (Bartoń, 2018). A Generalized Linear Mixed Model (GLMM) was constructed using the *lme4* package containing all parameters of interest for datasets A and D (Bates et al., 2015). A Generalized Linear Model (GLM) was constructed for datasets C and D because these models were constructed without random effects, as there were no repeat individuals. The GLM or GLMM was then dredged, i.e., all combinations of parameters were constructed and then scored and ranked using AIC. A delta AIC score was determined based on the AIC of the existing model minus the model with the lowest AIC score. Delta AIC scores larger than 7 typically indicate that the model is uninformative, so these were discarded (Burnham & Anderson, 2003; Burnham et al., 2011). Coefficient averages were then calculated across all remaining models. Any parameter whose CI includes zero is considered uninformative (Lane, 2007). The sum of AIC weights measures relative variable importance by estimating the probability that a parameter is included in the best IT model (Giam & Olden, 2016). All parameters with a score of 1 indicate the importance to the dependent variable of this parameter.

4.4 | RESULTS

4.4.1 | ML Approach

Accuracy and precision scores from logistic and RF models were compared to one another after the models predicted on the training_validation sets. Only training_validation scores are compared to avoid leakage. Leakage occurs when information outside of the training_vaidation is used to choose a model, such as looking at the final test score. The better model (whose score performed higher: Fig. 4.2) was chosen to run on the test set (Table 4.2).

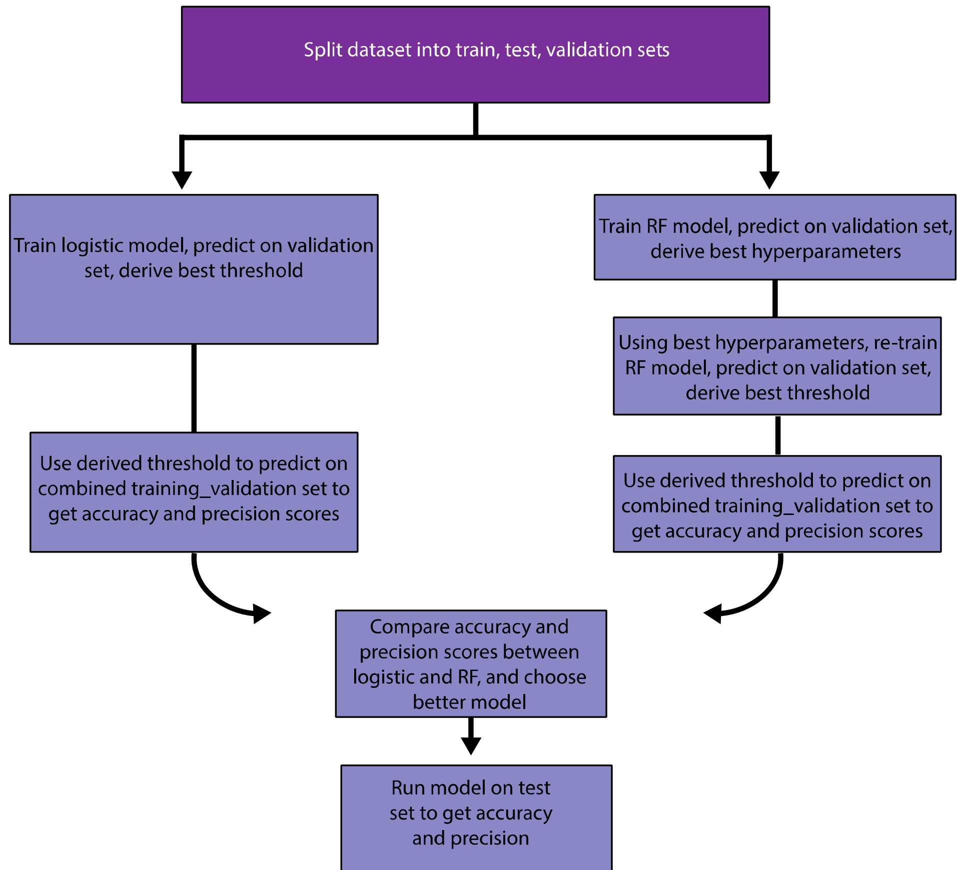

Figure 4.2. The specific machine learning pipeline used to predict reproductive outcomes in black-footed ferrets.

A baseline accuracy score was established for each dataset. The baseline accuracy score is the same for both the logistic and RF models because it is simply the score that would result from the maximal class being predicted for all outcomes.

For dataset A, RF was chosen over logistic because accuracy and precision scores for the logistic were low for both the default and custom thresholds compared to the RF custom threshold scores (Table 4.4). Though the high RF scores might indicate overfitting, this model contained the most parameters for estimation, which might also lead to such high scores.

For dataset B, a logistic was chosen over RF because the precision score for the custom threshold was quite high, which indicates that there is a good chance that the model would do a

good job of predicting whelping, even if accuracy was not as high as the RF (Table 4.4). Therefore, even though the RF scores were much higher, there is a higher chance on this small dataset that overfitting led to those high scores.

For dataset C, a RF using the custom threshold was chosen. The logistic regression default and custom threshold scores had higher accuracy, but precision was not as high as the RF custom threshold (Table 4.4). Additionally, although the RF default threshold also had higher accuracy, the superior precision score of the custom threshold is a strong indicator that the model may be good at predicting whelping rates.

For dataset D, all scores were not much higher than baseline, indicating that the model is very unlikely to be predictive (Table 4.4).

Table 4.4: Machine learning accuracy and precision scores for predicting reproductive outcome in black-footed ferrets across four datasets using derived and custom thresholds on the training_validation set.

				Default threshold scores			Custom threshold scores		
	Algorithm	Baseline accuracy score	Default threshold	Accuracy on training_val data	Precision on training_val data	Custom threshold	Accuracy on training_val data	Precision on training_val data	Model choice
Database A	Logistic	0.524	0.50	0.640	0.635	0.495	0.640	0.631	Random Forest, custom threshold
	Random Forest			0.967	0.947	0.511	0.968	0.958	
Database B	Logistic	0.606		0.727	0.762	0.764	0.727	0.958	Logistic, custom threshold
	Random Forest			0.955	0.930	0.702	0.924	1.0	
Database C	Logistic	0.507		0.696	0.692	0.621	0.691	0.778	Random Forest, custom threshold
	Random Forest			0.728	0.722	0.635	0.682	0.828	
Database D	Logistic	0.620		0.653	0.561	0.701	0.623	0.667	Random Forest default threshold
	Random Forest			0.662	0.741	0.769	0.620	0.0	

The test set for each dataset was then run using the algorithm and threshold that gave the best scores on the training_validation set. On the test set, dataset A, B and C performed better than random i.e. each model had a predictive accuracy higher than baseline (Table 4.5). Dataset D performed worse than baseline. Dataset B performed the best, indicating that sperm parameters helped contribute to the higher predictability of this model. Dataset A (most encompassing model) performed second best, and Dataset C performed third best, indicating that past breeding outcomes for males to some degree influence the ability to predict breeding outcome of future events. Dataset D performed worse than baseline, indicating that past female breeding outcomes do not help predict future breeding outcomes.

Table 4.5: Machine learning accuracy and precision scores for predicting reproductive outcome in black-footed ferrets across four datasets on the test set.

Dataset	Algorithm	Threshold used for test set	Baseline accuracy score	Accuracy on test data	Precision on test data	Accuracy subtracting baseline
A	Random Forest, custom threshold	0.511	0.524	0.643	0.606	0.119
B	Logistic, custom threshold	0.764	0.606	0.824	0.889	0.218
C	Random Forest, custom threshold	0.635	0.507	0.618	0.533	0.111
D	Random Forest default threshold	0.500	0.620	0.610	0.0	-0.010

4.4.2 | Statistical modeling approach

4.4.2a | Dataset A

For the most encompassing dataset, a GLMM using the IT technique was built to understand factors influencing reproductive outcomes. The parameters tested included sire ID and dam ID as random effects and sire *F*, dam *F*, kinship, dam and sire age, dam and sire cumulative count, facility and breeding year as fixed effects (Table 4.3). The dependent variable in this and all datasets was a binary reproductive outcome (whelped or DNW).

Using sum of AIC weights, breeding year, sire age, and sire cumulative count had full support (a sum of weights of 1) in 27 models. The model-averaged coefficients indicate that both breeding year and sire age have significantly negative impacts on reproductive outcome, while sire cumulative count has a significant positive impact (Table 4.6). Even after adjusting the significance threshold for the number of predictors (testing $p \leq 0.006$), breeding year, sire age and sire cumulative count are still significant.

Table 4.6. Information Theoretic model summary for Dataset A in determining factors that influence reproductive outcome in the black-footed ferret.

Factor	Estimate	Adjusted Std. Error	z value	Pr(>|z|)
(Intercept)	**-0.354**	**0.131**	**2.701**	**0.007**
Breeding Year	**-0.493**	**0.116**	**4.259**	**0.000**
Dam Age	-0.202	0.161	1.253	0.210
Dam cumulative count	-0.223	0.163	1.368	0.171
Dam F	-0.164	0.126	1.299	0.194
Sire Age	**-0.706**	**0.110**	**6.418**	**< 2e-16**
Sire cumulative count	**0.559**	**0.117**	**4.774**	**0.000**
Kinship	0.016	0.047	0.350	0.727
Sire F	0.010	0.060	0.158	0.875

Factor	Estimate	Adjusted Std. Error	z value	Pr(>|z|)
Facility: USFWS's National Black-footed Ferret Breeding Center	0.005	0.079	0.064	0.949
Facility: Louisville Zoological Gardens	0.039	0.210	0.185	0.853
Facility: Phoenix Zoo	-0.007	0.116	0.057	0.955
Facility: National Zoo's Smithsonian Conservation Biology Institute	0.034	0.165	0.207	0.836
Facility: Toronto Zoo	-0.019	0.170	0.113	0.910

4.4.2b | Dataset B

For the dataset testing seminal traits, a GLMM was built including the parameters sire ID as the random effect and dam and sire F, kinship, dam and sire age, year of AI, sperm motility, forward progression and normal acrosome as fixed effects (Table 4.3).

Using sum of AIC weights, normal acrosome had full support in 122 models. The model-averaged coefficients indicate that normal acrosome had a positive significant impact on whelping success (Table 4.7). Even after adjusting the significance threshold for the number of predictors ($p \leq 0.006$), normal acrosome is still significant.

Table 4.7. Information Theoretic model summary for Dataset B in determining factors that influence reproductive outcome in the black-footed ferret.

Factor	Estimate	Adjusted Std. Error	z value	Pr(>|z|)
(Intercept)	0.334	0.334	0.998	0.318
Dam age	-0.469	0.433	1.084	0.278
Fresh/Frozen % Normal Apical Ridge	**1.326**	**0.476**	**2.788**	**0.005**
Fresh/Frozen % Motility	-0.351	0.481	0.730	0.465

Factor	**Estimate**	**Adjusted Std. Error**	**z value**	**Pr(>|z|)**
Sire Age	-0.288	0.383	0.752	0.452
Sire F	-0.231	0.372	0.621	0.535
Year of Artificial Insemination (AI)	-0.245	0.376	0.653	0.514
Fresh/Frozen Status	0.128	0.313	0.411	0.681
Dam F	0.063	0.335	0.186	0.852

4.4.2c | Dataset C

For the dataset that includes past male outcomes, a GLM was built with parameters including facility, sire *F*, sire birth year, outcome 1 and outcome 2 as fixed effects. The dependent variable was outcome 3 (whelped or DNW).

Using sum of AIC weights, outcome 1 and outcome 2 had full support in 5 models. The model-averaged coefficients indicate that both a successful whelping outcome during the first and second breeding event has a positive impact on the third reproductive outcome (Table 4.8). Even after adjusting the significance threshold for the number of predictors ($p \leq 0.005$), outcome 1 and outcome 2 are still significant.

Table 4.8. Information Theoretic model summary for Dataset C in determining factors that influence reproductive outcome in the black-footed ferret.

Factor	**Estimate**	**Adjusted Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	**152.177**	**69.553**	**2.188**	**0.029**
Outcome1: Whelped	**1.066**	**0.276**	**3.868**	**0.000**
Outcome2: Whelped	**1.134**	**0.277**	**4.096**	**0.000**
Sire Birthyear	**-0.076**	**0.035**	**2.202**	**0.028**
SireF	-0.504	4.323	0.117	0.907
Facility: USFWS's National Black-footed Ferret Breeding Center	0.004	0.078	0.045	0.964

Factor	Estimate	Adjusted Std. Error	z value	Pr(>|z|)
Facility: Louisville Zoological Gardens	0.002	0.124	0.019	0.985
Facility: Phoenix Zoo	-0.014	0.154	0.091	0.927
Facility: National Zoo's Smithsonian Conservation Biology Institute	-0.009	0.106	0.088	0.930
Facility: Toronto Zoo	0.022	0.206	0.108	0.914

4.4.2d | Dataset D

For the dataset that includes past female outcomes, a GLM was built with parameters including facility, dam *F*, dam birth year, outcome 1 and outcome 2 as fixed effects. The dependent variable was outcome 3 (whelped or DNW).

Using sum of AIC weights, outcome 2 had full support in 16 models. The model-averaged coefficients indicate that a successful whelping outcome during the second breeding event has a positive impact on the third reproductive outcome (Table 4.9). Even after adjusting the significance threshold for the number of predictors ($p \leq 0.005$), outcome 2 is still significant.

Table 4.9. Information Theoretic model summary for Dataset D in determining factors that influence reproductive outcome in the black-footed ferret.

Factor	Estimate	Adjusted Std. Error	z value	Pr(>|z|)
(Intercept)	29.901	50.679	0.590	0.555
Outcome1: Whelped	0.168	0.230	0.730	0.465
Outcome2: Whelped	**1.054**	**0.225**	**4.679**	**0.000**
Dam Birthyear	-0.015	0.025	0.610	0.542

Factor	Estimate	Adjusted Std. Error	z value	Pr(>|z|)
Facility: USFWS's National Black-footed Ferret Breeding Center	0.169	0.335	0.505	0.613
Facility: Louisville Zoological Gardens	-0.213	0.600	0.355	0.723
Facility: Phoenix Zoo	-0.269	0.627	0.428	0.668
Facility: National Zoo's Smithsonian Conservation Biology Institute	0.259	0.455	0.569	0.569
Facility: Toronto Zoo	0.302	0.724	0.418	0.676
Dam F	-1.877	7.492	0.251	0.802

4.5 | Discussion

Many species under intensive management have years of data collected on behavior, lineage, geographical location, health parameters, management decisions, reproductive outcomes and more. ML has the potential to utilize this abundance of information to make predictions about the future by learning trends within the dataset and using patterns it discovers (Bzdok et al., 2017). Here, we were able to use ML methodologies to help predict reproductive outcomes to varying degrees of accuracy based on the years of studbook data and reproductive records. Additionally, we were able to derive what factors most impact reproductive success for each dataset.

The most successful model, using a combination of individual biographical data and seminal characteristics from AI events (Dataset B), allowed us to predict reproductive outcome correctly 82% of the time, and accurately predict a "whelped" outcome 89% of the time. This result allows managers to have some level of confidence that seminal characteristics from a

specific male will predict whether he will sire a litter, regardless of the female chosen for the mating event. We know that the model's superior predictive ability is in large part due to the sperm metrics because the other factors included in the model (*F*, age, year, etc.) were included in a separate model (Dataset A) without sperm characteristics that did not perform as well as this model. Our model-based statistical analysis found somewhat similar results. A higher percent normal acrosome had a significant positive effect on reproductive outcome (Table 4.7). While sperm motility and forward progression were not found to be significant determinants of reproductive outcome, this finding still shows the importance of sperm quality – specifically the acrosome – on fertility. An intact and functioning acrosome is necessary for sperm capacitation and fertilization (Hirohashi et al., 2018). A recent study in ferrets found similar conclusions about the importance of sperm characteristics, where sperm traits positively influence litter production and size (R. Santymire et al., 2018). Interestingly, year of artificial insemination was not significant in our study, indicating that the artificial insemination procedures over the years have not been variable enough to significantly impact reproductive outcome. However, increasing inbreeding over time may have caused the decrease in sperm quality, thereby contributing to the decline in reproductive success over time (Santymire et al., 2018) The impact of increased inbreeding on sperm quality and thereby, fertility, has been confirmed in a number of species (Fitzpatrick et al., 2009; Pukazhenthi et al., 2006). For example, in Mexican gray wolves (*Canis lupus baileyi*), high levels of inbreeding were inversely correlated with percent of motile sperm and percent of normal sperm morphology (Asa et al., 2007).

Compared with the ML results of Dataset B, Dataset A, which did not include sperm parameters, was able to predict reproductive outcome correctly 64% of the time and accurately predict a "whelped" outcome 60% of the time. While this was an improvement on the baseline

score by 11.9 points (Table 4.3), it highlights the significant increase in predictability power that sperm parameters add to a model. However, the predictors included in this model such as genetic relatedness, past breeding experience and management years suggest that these types of considerations are all important for predicting breeding outcomes. The statistical IT approach for Dataset A found that sire cumulative count, breeding year and sire age (Table 4.6) were significant factors on breeding outcome. Our model found that as year increased, the chance for whelping decreased, indicating that in more recent years, whelping success has become rarer. This may be due to the increasing *F* over the years. The statistical results also indicate the more pairings a male has had, the higher his chance is for a reproductive success in the future. Reproductive success in several bird species has been shown to be tied to breeding experience (Limmer & Becker, 2010; Pitera et al., 2021; Pyle et al., 2001). In contrast, sexual experience in male mice did not determine reproductive outcome (Thonhauser et al., 2019).

The results from Dataset A also indicated that dam experience is not a significant factor for reproductive success, which contrasts with what has been observed in other species (Bauman et al., 2019; Saunders et al., 2014). In the case of the ferret, male experience seems to be the determining factor regardless of female experience. Also in contrast to many findings in mammals and birds (DeVries et al., 2016; Ricklefs et al., 2003), female age had little power to predict reproductive success in ferrets. This is largely due to the recovery program, which discovered that dams over the age of 3 had reduced reproductive success. Therefore, the program breeds mostly females from 1 to 3 years of age (Graves et al., 2019).

The ML model that tested whether previous sire outcomes influenced future reproductive events (Dataset C) also proved to be predictive. This model predicted reproductive outcome correctly 62% of the time, and accurately predict a “whelped” outcome 53% of the time. This

was an improvement on the baseline accuracy score by 11.1 points (Table 4.3). While less predictive than Dataset B's model, this model indicates that combined male-only factors positively influence reproductive outcome (Table 4.8). When these same factors were tested in a statistical framework, a whelping event for outcome 1 and outcome 2 significantly positively impacted future reproductive events (Table 4.8). Thus, males who bred successfully their first and second time had a higher chance of successfully breeding their third time. This parallels the findings from Dataset A's model and findings in other species that male experience is critical for reproductive success (Markussen et al., 2019).

The fourth ML model that focused on female traits (Dataset D) was not at all predictive and performed worse than baseline (Table 4.3). This mirrors findings in earlier models that female-specific traits are not necessarily predictive of future reproductive outcome, and that in the ferret, male characteristics are more important for accurately predicting reproductive successes or failures. In Dataset D's statistical framework, however, outcome 2 was found to have a significant impact on the third reproductive outcome (Table 4.9). Therefore, if a female bred successfully her second time she was more likely to successfully breed her third try. Moreover, a female that did not breed successfully her first time is not less likely to succeed in the subsequent breeding events. This somewhat contrasts with what is observed in other mammals (Casimir et al., 2007).

An important finding in all models is that facilities never significantly impacted reproductive outcome. This indicates that the captive breeding program is successfully managing the separate populations as one.

We had hypothesized that kinship between dam and sire, as well as F, would have significant influence on breeding outcome, but they did not. It seems the population is managed

so rigorously that the spread amongst kinship and *F* (range: 0 - 0.3574; Marinari et al., 2021) are too small to detect any significant influence on reproductive outcomes. Percent breeding success for similarly inbred red wolves (*Canis rufus*; range: 0-0.5 and above) significantly decreased as inbreeding coefficient increased (Bauman et al., 2019). For example, male wolves with $F = 0$ had a 59% chance of reproductive success but males with *F* at a range of 0.2-0.299 had a 37.1% chance of reproductive success (Bauman et al., 2019). The fact that we did detect such differences in ferrets may indicate that the *ex situ* breeding program has managed *F* to the point where other factors are more influential for breeding outcome.

It is clear from our analysis and data that in black-footed ferrets, male characteristics are largely responsible for reproductive outcome. Seminal traits are highly predictive of reproductive success, and some predictive power comes from a combination of traits such as *F*, kinship, age, breeding year, kinship and past reproductive successes. With increasing access to entire genomes for individuals, we can use this information to help improve the diversity of the overall population and thereby evaluate how it improves reproductive success. The recent first ever cloning of a black-footed ferret provides new horizons in assessing the impact of new cell lines in the gene pool and what this will do for reproductive rates (Sandler et al., 2021).

CHAPTER FIVE | Conclusion and future direction

Human-caused changes in the environment have forced flora and fauna globally to either adapt quickly or disappear (Sandra et al., 2019). Species that have co-evolved with highly specific ecosystem dynamics find themselves in completely new, uncharted environments suddenly, making it very difficult to adapt (Davidson et al., 2009; Davies et al., 2004). The evolutionary clock does not respond in such short time increments (Doak & Morris, 2010; Quintero & Wiens, 2013). For many species, it has been survival of the luckiest.

One of the world's most endangered mammals, the black-footed ferret, coming from one of the world's most endangered ecosystems, the prairie, is a textbook case for anthropogenic-caused population decline (Miller et al., 1996; Ricketts et al., 1999). Though captive breeding successfully saved the ferret from extinction, inbreeding depression continues to impact their reproductive success and chances for recovery (Miller et al., 1996; Santymire et al., 2018)(Miller et al., 1996; R. Santymire et al., 2018)(Miller et al., 1996; R. Santymire et al., 2018)(Miller et al., 1996; R. Santymire et al., 2018)(Miller et al., 1996; R. Santymire et al., 2018)(Miller et al., 1996; R. Santymire et al., 2018)(Miller et al., 1996; R. Santymire et al., 2018)(Miller et al., 1996; R. Santymire et al., 2018)(Miller et al., 1996; R. Santymire et al., 2018) . However, the fact that wild ferrets, who are descendants of the captive ferrets, have improved reproductive health (Livieri, pers. comm), is an interesting and promising avenue for exploration. Their improved reproductive traits suggest that environmental-dependent inbreeding depression (EDID; Cheptou & Donohue, 2011) is taking place between captive and wild ferrets i.e. though captive ferrets are similarly inbred as wild ferrets, the negative effects of inbreeding are exacerbated by the captive environment.

My dissertation work has provided substantial evidence for EDID in the ferret, and support for the idea that captive environmental conditions can be altered to lessen the effects of inbreeding, evidenced by improvements in traits related to fertility through experimental diets. In Chapter Two, lower levels of DNA damage in second-generation vitamin E ferrets compared with first-generation vitamin E ferrets suggest that antioxidant supplementation has potentially protective effects for mature sperm DNA integrity. The mechanism driving this may be transgenerational, since all second-generation ferrets had mothers on vitamin E supplementation during conception and pregnancy. However, the "silver spoon" hypothesis (Graffen, 1988) is also a potential mechanism for the lower sperm DNA damage levels in second-generation ferrets. This theory posits that favorable conditions early in life will positively affect the fitness of the individual later in life. Second-generation vitamin E ferrets may have improvements over first-generation vitamin E ferrets in their DNA integrity because they were supplemented with antioxidants during development, which was thereby carried throughout their reproductive years. There was also evidence that ferrets overall in 2021 had lower levels of sperm DNA damage, indicating that the TOR diet's increased fat composition due to COVID meat limitations may have actually positively impacted sperm health overall. This work also validated the use of sperm DNA damage as a fertility metric, since it was found to be correlated with sperm parameters that are predictive of fertility.

Further, the finding in Chapter Two that seminal plasma total antioxidant capacity (TAC) is negatively correlated with DNA damage also provides support for the fact that seminal plasma antioxidant content is protective of sperm cells. Additionally, the finding that second-generation vitamin E ferrets had lower TAC levels in blood serum than control and first-generation vitamin E ferrets suggests less demand for oxidative repair. This supports the idea that antioxidant

supplementation provides protective advantages during adulthood, though it is still to be determined whether benefits are conferred via the silver spoon hypothesis or transgenerationally. Future research should focus efforts on comparing TAC, sperm morphology and DNA damage levels for ferrets born to control ferrets but supplemented with vitamin E early in life, with second-generation vitamin E ferrets. This can help elucidate the mechanism of *how* antioxidants drive improvements.

Chapter Three, too, finds evidence of EDID taking place in the ferret through gene expression patterns. Wild ferret sperm, though genetically similar to captive ferrets, had functional enrichment in motility- and acrosome-related functions. We find evidence of this translating to improved sperm metrics that we determined in chapter two. For example, wild ferrets have significantly less damaged acrosome and apical ridge than control diet ferrets. It is possible that increased expression in genes related to these functions was translated to proteins contributing to these functions, thereby manifesting in the improved phenotypes we observe with wild ferrets. Functional enrichment in vitamin E compared to control ferrets told less of a cohesive story, but did show that pathways related to various cellular activities like ion binding, transmembrane movements, potassium channel activity activation, and olfaction were enhanced, suggesting that antioxidant supplementation had an impact on the system and was being processed. We did not find evidence of differential expression in genes related to protamines across diets, which may support my hypothesis that both captive and wild ferrets have similarly altered protamine gene expression, thereby making DNA more vulnerable, but that the wild ferret diet and environment keeps the vulnerable DNA more protected than the captive environment. This hypothesis, along with hypotheses surrounding other gene expression patterns

and how they contribute to sperm phenotype, need to be tested through proteomic work, which would show whether these transcripts are actually being translated into the phenotypes I discuss.

There was no enhancement of oxidative repair pathways in vitamin E ferrets, which may suggest that the lower levels of DNA damage in vitamin E ferret sperm may in fact be related to repair done by transcripts in seminal plasma, which we did not explore. Examining seminal plasma RNA transcripts would be an important next step in understanding the ferret sperm transcriptome, since it may express its own unique RNA profile across diet treatments and contribute its own functional enrichment for repairing damage (Jodar, 2019). Overall, characterizing gene expression in mature sperm in non-model organisms is rarely done, especially in threatened species, and my study is the third to do so (Ran et al., 2018; Schuster et al., 2016).

Chapter Four found that managers and researchers can be empowered in their decision-making processes by relying on routinely collected data in captive breeding programs (Bauman et al., 2019). If these data can be collated and standardized, it can be fed into machine learning algorithms that can help improve our understanding of the factors that actually positively predict reproductive success. This work confirmed that sperm parameters, and in particular acrosome-related parameters (related to sperm's ability to penetrate the oocyte), are the most important factors for predicting whelping success. Female factors were not found to be influential in determining reproductive outcome. Past reproductive success in males was also found to positively impact future reproductive events, confirming that proven males will more often succeed in the future. This overall suggests that young males who have higher sperm parameters will sire and continue to sire in the future. This knowledge can allow managers to make more informed decisions regarding pairing for mating during the breeding season, and also encourages

the Black-footed Ferret Recovery program to consider changing the standard diet overall, since the outlook for ferrets with lower sperm parameters is lower fertility rates. My previous chapters show that antioxidant supplementation does lessen demand for oxidative repair and even lowered sperm DNA damage levels, which can provide evidence to the BFF program that antioxidant diet is one method to achieve improved chances at reproduction for young males into their future. Wild ferrets showed even more evidence for these improvements, which might also encourage the program to consider altering the captive ferret's environment for increased fertility. While the carcass diet sample size was small, we did not find evidence that it emulated the improved sperm parameters that we observed in wild ferrets. The fact that the carcass diet that was most similar to the wild diet but did not show improved fertility metrics suggests that there is something in addition to but beyond the wild diet that makes the wild environment the best conditions for ferret reproductive health.

My modeling work also found that inbreeding coefficient, kinship, or facility were not important predictors of reproductive success, suggesting that the captive breeding programs have successfully managed various population as one, and have minimized the influence of inbreeding to the point that other factors are more influential for reproductive success. Since the goal of captive management programs are to increase breeding rates to eventually establish sustainable populations that can eventually be reintroduced back into the wild, tools such as the machine learning pipeline I created will become increasingly useful in helping to determine how to make this happen. Using such a pipeline in the future would benefit from genetic data, since this highly individualized perspective can greatly influence our ability to predict outcomes in general.

Overall, although antioxidant supplementation did show a decline in second-generation vitamin E DNA damage levels over first-generation, evidence is still inconclusive as to whether

the antioxidant supplementation is the best way to improve reproductive health. There was no difference in reproductive outcomes across diet study, indicating that antioxidants, at least across the generations tested, did not impact the ability to whelp. Additionally, gene expression patterns across diets were relatively similar. While there were enhancements in certain cellular functions in vitamin E ferrets, there was no conclusive evidence that the cellular pathway enhanced contributed to improved fertility metrics. There was much more expression-level evidence that the wild diet and conditions are optimal for ferrets. It seems that the captive breeding programs should focus their energies on recreating a wild setting as much as possible, in order to increase sperm metrics and chances at improved fertility rates.

Beyond exploring whether the positive impact of vitamin E is delivered via the "silver spoon" hypothesis or transgenerationally, as well as delving into proteomic analysis to see whether expressed genes are actually being translated, future studies should focus on the impact of the gut microbiome in the black-footed ferret. The microbiome, the collection of microorganisms living in an environment, has been shown to impact everything from digestion to behavior, organ development and reproduction (Dinan et al., 2015; Erny et al., 2015; Wang & Xie, 2022). Wild microbiomes are especially important to characterize in order to understand the natural variation that individuals experience and how this impacts overall health, especially since the captive environment significantly changes the microbiome (Hird, 2017). Characterizing the microbiome across diets, environments and fertility status can provide us a look at how bacteria influence gene expression (Nichols & Davenport, 2021) and how this can impact fertility parameters. Therapeutics have been developed to alter the microbiome for improved health outcomes (McQuade et al., 2019; Ribeiro et al., 2017) and this could potentially be an avenue towards improving fertility in the black-footed ferret.

Beyond the microbiome, the black-footed ferret has recently been cloned and this can provide the population with genetic diversity that has been lost for decades (Sandler et al., 2021). Further genetic exploration of the impact that Elizabeth Ann’s genes may have on future generations will allow us to assess whether cloning should be conducted again and how frequently to help save this species from extinction.

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