Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Anneli Steen Johansen

A tale of stress, reproduction, survival and climate change

Case study of the Norwegian Arctic fox (*Vulpes lagopus*) and measurement of glucocorticoids

Master's thesis in Biology

Supervisor: Clare Stawski, Arild Landa, Anne-Mathilde Thierry, Nina E. Eide

August 2020

Photo & edit: Anneli S. Johansen

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Acknowledgement

This master thesis was written for Department of Biology at the Norwegian University of Science and Technology (NTNU), in collaboration with the Norwegian Institute of Nature Research (NINA). Big thanks to my supervisors who have endured through this long adventure. Thanks to Arild Landa for constructive guidance in the writing process and keep reminding me to not forget "the flow". Thanks to Nina E. Eide for being patient and understanding .Thanks to Anne-Mathilde Tierry for believing in me to handle labwork without supervision. Thanks for helping with troubleshooting and helping me finding alternative solutions, and exploring 'ELISAtools' with me. Thanks for taking the time every once in a while to help me even long after you changed jobs. Last but not least, thanks to Clare Stawski for accepting me as one of her students when she first started at NTNU. Thanks for the support and I'm happy I got to work with you. Good luck with your bats!

Additional thanks to Luis and Craig for making the time stepping by as short term supervisors to help me. Thanks to Christophe Pelabon and all PhD students who provided clarification regarding R and statistics. I had a great time doing field work at Sæterfjellet Avlsstasjon and at Dovre. Happy to share my first time hiking for days and sleeping outside while looking for foxes with Elisa Keeling Hempholl and Leila S Berg, even though that expedition was short lived. I'll never forget walking for miles through an unannounced rainstorm and avoiding an army of moskox in just one day. I'm grateful to everyone at NINA who I got to know during my time in the lab, for sharing laughs and nice conversations. From Andrea Miller & Co at the virus lab to Line Birkeland Eriksen & Co at the genetic lab who lend me their equipments and helping me whenever they could. Anikó Hildebrand and Heidi Myklebost for lending me their labspaces. And of cource Kristine Ulvund for fast replies when I needed data extracted from their database and for being around the corner if I ever needed something.

Biggest warmest thanks to my parents who I can call any time.

Abstract

The Arctic tundra is under pressure from climate change; climate sensitive species like rodents might have their cycle fluctuation disrupted as a result. Rodents are for most arctic carnivore and omnivores an essential part of their food source. When rodent populations crash the population of their predators follow. Starvation and other stressors, e.g. weather, activate the HPA-axis releasing e.g. cortisol. Physiological measurements can thus uncover the state of the population before it is reflected in their population dynamics. Measuring stress hormones from hair is a non-invasive method of sampling and it eliminates the risk of effecting stress levels during the procedure. Glucocorticoids (GC) are widely used for stress estimations, they influence several biological functions like metabolism, development, immune system, reproduction. Elevated or low GC levels thus have the potential to affect fitness. This project aims to contribute to expanding the field of conservation physiology and provide results which can enrich discussion of GC's possible link to fitness. The Arctic fox (*Vulpes lagopus*) is critically threatened in Fennoscandia and several of the threats to its decline are believed to be rooted to climate change. Uncovering their level of stress, how this correlates to fitness, and what stressors might result in a higher level of stress is important for future conservation work. I used fur of Arctic foxes, mainly pups, from the wild and a breeding station collected throughout summers 2014-2018. The hairs were washed and cortisol extraction happened according to Meyer (2014), the hairs were cut by hand, and I used ENZO's Cortisol ELISA kit (2016) to perform the assay.

According to my results cortisol level does not correlate with survival or reproductive success in Norwegian Arctic fox. Juveniles living in the wild show a greater link between their cortisol and environmental predictors; temperature deviation and rodent abundance, than juveniles in a breeding station. Wild litters are positively correlated with rodent abundance and temperature deviations, implying 5°C above normal summer temperatures works in Arctic foxes favor at the moment. In years with few litters in the population cortisol level of wild pups increases. This might be a result of low rodent abundance which ensues starvation and/or competition within litter. Either the link between GC and fitness are weak in general (supported by many contradicting findings) or the Arctic fox are GC-resistant and not stressed enough to have fitness affected.

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Table of contents

1.0 Introduction

Changes of environmental temperature are expected to put pressure on wildlife as these changes happen faster than many species ability to adapt. Estimation shows that 47% of mammals will live in a habitat with temperatures above their thermoneutral zone (TNZ) by 2080; arctic and boreal zones are the biomes most effected by climate change, especially at high altitudes (Mitchell et al., 2018, Post et al., 2009). The arctic tundra is a fragile system as food sources are already scarce, and food is an important environmental factor influencing reproduction in mammals (Elmhagen et al., 2000). Climate change can also include decreased snow cover and runoff, plus an increase of fires and drought during the summertime (Climate, 2020). If environmental changes happen faster than species' ability to adapt, the population might enter a vortex that leads to local extinction. Loss of a keystone species is likely to trigger a cascade of secondary extinctions, rare species with a strong interaction with many consumers can result in the largest number of secondary extinctions (Christianou and Ebenman, 2005). Conservation physiology is a new field using physiological methods to predict or understand how organisms respond to stressors, e.g. environmental changes, in order to improve conservation management (Cooke et al., 2013).

1.1 Stress response and how to measure it

These changes can be referred to as stressors as they create a stressful environment for the native species. Physiological stress is an adaptive physiological response (behavioral and physiological) to rehabilitate homeostasis after stimulus of stressors (Dantzer et al., 2014). This is vital because homeostasis is the state in which organs, cells, enzymes and proteins function; deviations from this could lead to changes of membrane fluidity, disrupt protein structure, and activate apoptosis (Hansen, 2009). Hypothalamus is the judge and the conductor of all this, through the nervous system it receives signals about the condition of the internal and the external. Then it applies suitable responses in the form of releasing hormones that initiate a cascade of hormone secretion. Hormones that are majorly involved in stress responses are glucocorticoids (GC) (Gilg et al., 2012, Whirledge and Cidlowski, 2010). GC (e.g. cortisol) influence approximately 10% of the genome, which include genes authorizing them to regulate many different systems (Breuner et al., 2013); GC have the ability to withdraw energy and substrates from reserves and direct them to exercising muscles, stimulate immune function, suppress sexual behavior, decrease appetite whereas cognition is improved (Sapolsky et al., 2000). Excessive stress over time cumulates negative impacts on health, both physical and psychological, e.g. regulate cardiovascular systems, decrease immune activation, affect neuroendocrine structures and central nervous systems (Lee et al., 2015, Whirledge and Cidlowski, 2010). Thus, changes of GC levels can change several physiological responses through the hypothalamic-pituitary-adrenocortical (HPA) axis and hypothalamic-pituitary-gonadotroph (HPG) axis (Figure 1). Cortisol can be extracted from blood, salvia, hair, feathers, and feces, which makes

this method widely used in wildlife biology (Stalder and Kirschbaum, 2012). The mechanism behind incorporation of cortisol into hair is unknown, one hypothesis of incorporation is passive diffusion from blood, sweat and/or sebum (Meyer and Novak, 2012). However usage of cortisol measurements from hair is still widely used as there is evidence supporting that GC production occur in hair follicles and hair provides a long-term index of HPA activity compared to the other sources (Meyer and Novak, 2012, Ito et al., 2005, Stalder and Kirschbaum, 2012, Accorsi et al., 2008).

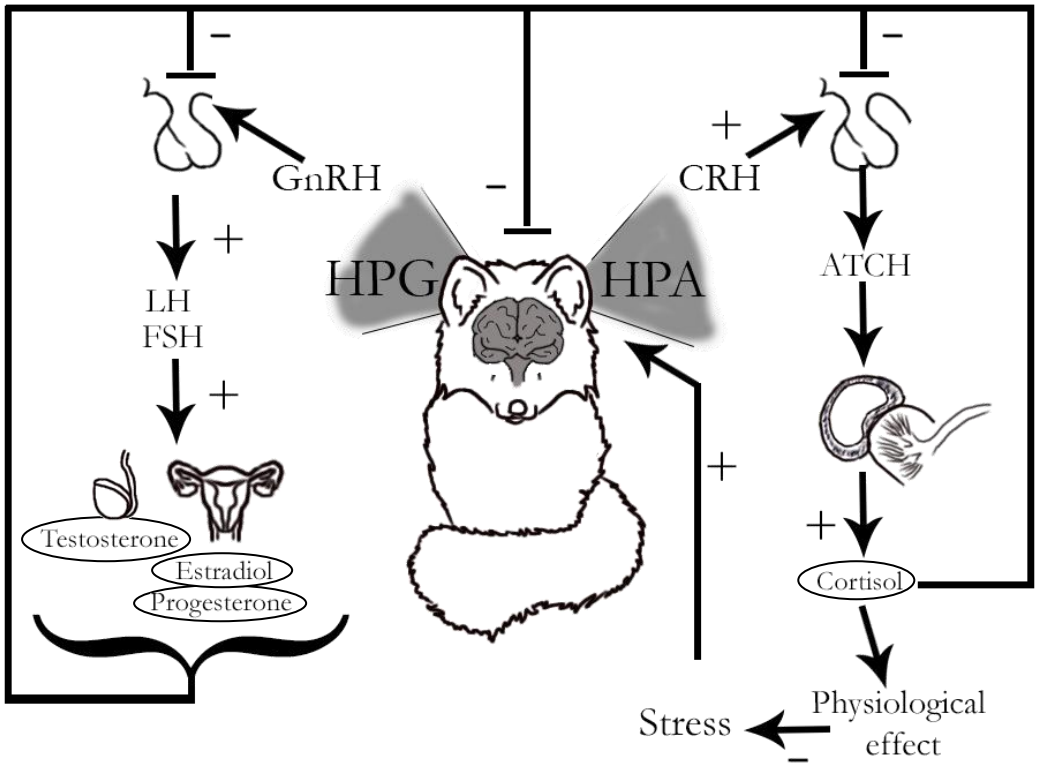

Figure 1. HPG (left) start in hypothalamus in which Gonadotropin-releasing hormone (GnRH) stimulate anterior pituitary gland to release the gonadothropes Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH) which stimulate production of sex hormones in the gonads. A negative feedback goes back to pituitary gland and hypothalamus. HPA (right) also start in hypothalamus, Corticotropin-releasing hormone (CRH) stimulate anterior pituitary to secrete the hormone Adrenocorticotropic hormone (ATCH) which stimulate production of cortisol in the adrenal gland. Cortisol initiate physiological effects that lead to stress responses. Cortisol also has a negative feedback on the pituitary gland and hypothalamus (Whirledge and Cidlowski, 2010). Physiological effect include changes of cognition, reproduction, immune system, muscle and cardiovascular flow. Plus and minus signs illustrate positive and negative feedback respectively. Steroid hormones secreted by HPG and HPA pathways are circled.

Ecological stress physiology is relatively new, and more research is needed to understand how cortisol relates to stress. There is a debate concerning the sufficiency of total cortisol as an indication of stress as cortisol exists in two forms; free and bound (about 90% GC) (Breuner et al., 2013). Thereof free cortisol is hypothesized to be the only incorporated from the bloodstream to hair (Davenport et al., 2006, Russell et al., 2012), though I could not find experimental research directly testing or confirming this. Three hypotheses describes the relationship between the two forms; total hormone hypothesis assumes both free and corticosteroid binding globulin (CBG) are biologically active. Reservoir hormone hypothesis states CBG is stored until needed. Free hormone hypothesis assumes

only the free form of cortisol is biologically active and CBG makes cortisol unusable. Studies seems to support reservoir- and free hormone hypotheses which means the biological significance of measuring total cortisol cannot simply be discarded (Breuner et al., 2013). Even if hair cortisol consist mostly of free cortisol, then hair cortisol concentration can be influenced by changes of circulating CBG (Davenport et al., 2006). After 30-60 min of stress the release of circulating CBG decreases, resulting more free-GC that can be damaging (Breuner et al., 2013). More research is also needed on the link between cortisol in hair and other indicators of welfare e.g. fitness. Studies have shown contradicting results between cortisol and fitness (Yamanashi, 2018). Regardless, this method to assess stress has potentials.

1.2 How stress affects individual fitness

Previous research has shown a possibility for negative correlation between stress and survival (Jeannin, 2016), and between stress and breeding (Bonier et al., 2009). With the assumption that high GC levels indicate stressful environments (Bonier et al., 2009). There are several studies of long-term stress affecting gonadal function, but the effect on wild animals are not known. Different sexes have different gonadal responses to stress; for example in males testosterone production and spermatogenesis are decreased, even testes growth are affected (Bartke et al., 1977, Whirledge and Cidlowski, 2010). In one instance during a drought, Serengeti wild male baboons (*Papio anubis*) had a 60% decrease of basal testosterone after becoming stressed from using more time foraging-(Sapolsky, 1985). Major levels of stress can also make it hard to carry out the act of mating by resulting in erectile dysfunction, as erections are dependent on a balance between hormonal, neurological, vascular, psychological and cavernosal factors (Agarwal, 2006).

For females stress can have an even bigger consequence for reproductive success; it can effect fertilization, development and birth. GC has many roles in ovary physiology and work only at certain precise GC concentrations even a slightest deviation from normal can have an effect. Female mammals generally have a higher basal GC compared to males because of their estrogen and androgen levels (Whirledge and Cidlowski, 2010, Reeder and Kramer, 2005). During chronic stress ovulation does not take place (Suter and Schwartz, 1985). If chronic stress occurs after a fetus has been conceived there is a danger of miscarriage due to low progesterone concentration (Arck et al., 2007). Being pregnant costs a lot of energy and makes the female vulnerable for predation and pathogens, if there are limited resources in the environment the mother might suffer and offspring might not survive either (Costa and Sinervo, 2004). However, an increase of GC, not as intense as chronic stress, is preferable during ovulation as GC can be anti-inflammatory and limit ovarian inflammatory process (Whirledge and Cidlowski, 2010).

A mother's past experiences might affect the development of future offspring. One phenomenon is called metabolic imprinting/programming, from which offspring is being subjected to prenatal stress

(e.g. malnutrition), which can cause permanent changes to its physiology (Dyer and Rosenfeld, 2011, Hanley et al., 2010). Alternations might happen within neurodevelopment (cognition and neural mechanisms) and immune modulation to name a couple. Laboratory experiments on rats indicate female fetuses are more vulnerable for prenatal stress at the end of pregnancy (Welberg and Seckl, 2001), this could gradually decrease the quality of females in the population. Changing the physiology of a fetus based on the hormones it receives from its mother (through cardiac circulation and placenta) is an evolutionary adaptation to prepare the offspring for the environment that awaits it on the outside. The brain becomes less sensitive to GC and needs more to stop secretion of GC resulting in higher basal GC levels (Matthews, 2000, Edwards and Boonstra, 2016). However, when the environment changes again there is a mismatch between unnecessary high GC level and the level required by the environment, resulting in the offspring potentially developing diseases like metabolic syndrome (Edwards and Boonstra, 2016). After birth the offspring is continuously exposed to stress and an elevated GC concentration in the circulation continues to affect their development and health (Giustina et al., 2008, Van De Ven et al., 2006). Experiments has shown pups in larger litters experience competitive interaction between siblings and have higher cortisol levels than pups in small litters (Fey and Trillmich, 2008). Findings show that vertebrates with high stress exposure as a juvenile have a lower chance of surviving and reproducing successfully (Blas et al., 2007).

Stress and elevated GC are linked to immunosuppression. This can lead to negative effects like autoimmune disease and vulnerability to other diseases and pathogens, potentially decreasing their survival. However controlled immunosuppression is an adaptive trait: it is hypothesized as a way to reduce cost of energy and nutrients used on immune system (control reproductive cost), or immune suppression can be a way to avoid hyperactivation which might lead to autoimmune disease (Raberg et al., 1998). However, problems occur when the immune suppression precedes below the baseline, researchers have found a decrease of immune function can go as much as 40-70% below baseline in humans (Dhabhar et al., 1995). These are some of the many ways long-term stress can decrease fitness in wild animals (Reeder and Kramer, 2005, Blas et al., 2007).

1.3 Major stressors in arctic/alpine habitats

In arctic and alpine habitats stressors can appear in many forms; e.g. food availability, temperature/extreme weather events, predators/competitors and human disturbance. Predation risk has the potential to cause chronic stress and deterioration of reproduction in wildlife (Boonstra et al., 1998). Previous research has also shown that rodent populations plays an important role for sustaining predator populations (Ims et al., 2008, Meijer et al., 2013). The dynamic between small rodents and predators in Fennoscandian is susceptible to changes (Henden et al., 2009a). Climate change is very much a threat in the arctic biome as it is regarded to be one of the biomes most affected by climate change. Arctic species are adapted to an arctic terrain and deviations from arctic climate could lead to stress (Post et al., 2009, Callaghan et al., 2011, Barua et al., 2011). Though many species might be

able to cope with direct effects of climate change (e.g. temperature and UV-B radiation) but indirect effects (e.g. competition, predation and fragmentation) are expected to have a stronger impact (Hof et al., 2012).

Around the arctic circle there are usually cyclic fluctuations of rodents and their predators, 3-5 years in Fennoscandia (Norrdahl, 1995). However, climate change can lead to reduced cycle, peak densities of lemmings and their geographic range (Hof et al., 2012, Gilg et al., 2009). Lemmings and voles are herbivorous mammals low on the trophic level, regulation of their population level is thus reflected in their predators population dynamic (Norrdahl, 1995, Gilg et al., 2009). In unpredictable environments life history strategies may have different optimum at different phases (Tannerfeldt and Angerbjorn, 1998) and predators with a strong preference for a prey species does not change its diet when the abundance of that species decreases (Elmhagen et al., 2000). For example, Gilg et al.'s (2009) study of areas in eastern Greenland had an absence of the 4-year lemming (*Dicrostonyx groenlandicus*) cycle since year 2000. Snowy owl (*Nyctea scandiaca*) and Arctic foxes (*Vulpes lagopus*) deserted areas with absence of high lemming densities, Gilg et al also saw a lower breeding success of long-tailed skua (*Stercorarius longicaudus*) and in one area snowy owl had not bred for 8 years. They concluded climate change was the force behind (Gilg et al., 2009). There are several hypotheses on what causes population cycles; abiotic, biotic intrinsic and biotic extrinsic (e.g. predator-prey interactions). The herbivore-predation interaction is considered to have the biggest effect on a regular cycle, and abiotic factors have not been able to explain seasonal cycle of every cases (Norrdahl, 1995). However, abiotic factors have a crucial impact on rodents, especially in environments with limited resources. It can alter a relatively stable cycle that the species have adapted to, to one more irregular. Research has been done for many decades and none of the hypotheses explaining population cycles can give a universal explanation of cycles everywhere. In recent years it is becoming more challenging for researchers to predict timing of cycles. For the rodent population to peak they are dependent on deep snow as protection from predators, which climate change reduces (Kausrud et al., 2008). Dry, hot summers would cause a drought in the mountains making vegetation whiter creating a lack of essential nutrition to support a high abundance of rodents. More frequent fires results in a drop in the population and could make it challenging for rodents to reach past level of abundance during their peak year (Selås et al., 2018).

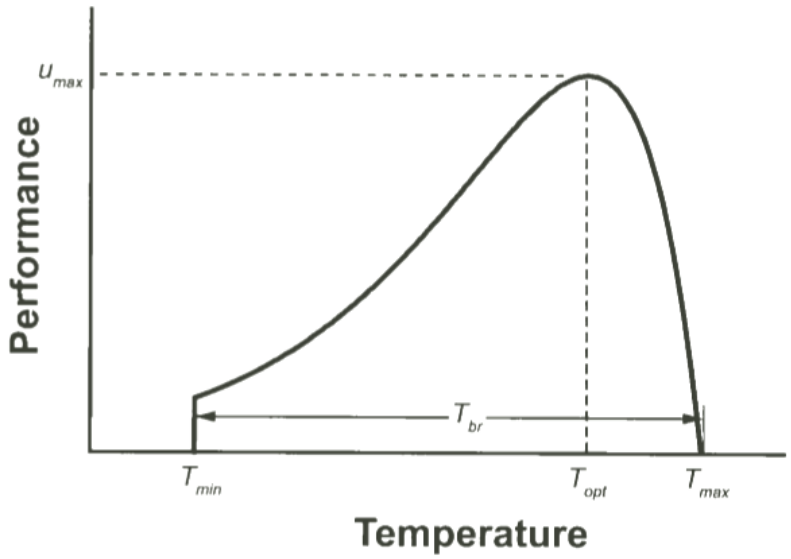

Figure 2. Theory of Performance Curve. Hypothetical animal performance/fitness decreases rapidly at a certain point of ambient temperature. Degree of thermal specialization (Tbr), maximum/minimum limit of performance (Tmax, Tmin), optimal temperature (Topt), temperature in which performance reaches a maximum rate (umax) (Gilchrist G. W. and F., 2008).

Environmental stress can be inflicted by high ambient temperatures, solar radiation, wind speed and humidity (Silanikove, 2000). Within TNZ mammals can maintain a stable balance between heat loss and heat production without activating mechanisms to control core temperature (e.g. evaporative cooling and metabolic processes) (Mekjavic and Eiken, 2006). Cooling reactions like panting and sweating can be sustainable but depends on resources to maintain these responses as they results loss of electrolyte and fluid, which could be lethal in excessiveness (Hansen, 2009, Mitchell et al., 2018). Core temperature is the temperature in which internal organs and bodily systems function at an optimal level and for most mammals core body temperature is around 35℃- 39℃ (Mekjavic and Eiken, 2006, Cengage, 2020). If the body temperature increases a few degrees over the set-point (maintaining homeostasis), membrane fluidity and protein structures are disrupted (Hansen, 2009). For example, cortisol affinity for CBG decreases as temperature increases. At 37℃ normalized free cortisol are at 100nM free cortisol, at 41℃ this has increased almost five times at low total cortisol, meaning there are more free-GC in circulation at higher body temperatures (Breuner et al., 2013). Hyperthermia might compromise reproduction by disrupting spermatogenesis, oocyte- and early embryonic development, lactation and placenta growth (Hansen, 2009, Mitchell et al., 2018) (Figure 2). Hyperthermia or hypothermia becomes dangerous when ambient temperature closing in on the critical thermal maximum and minimum which makes ectotherms immobile and ambient temperature can be lethal over long exposure (Mitchell et al., 2018).

1.4 Study species Arctic Fox

Geographic range of Arctic foxes is predicted to decrease by 43% in 2080, 13% in Fennoscandia, because of temperature related variables e.g reduced prey availability (Hof et al., 2012). The Arctic fox lives in the Arctic tundra from North America to Eurasia, including Greenland and other arctic islands. Mating takes place in March-April and pups are born in May-June. The pups start to adventure outside the den after 3-4 weeks and becomes sexually mature during their first year (Eide, 2017). As a natural top predator of its habitat, they prey on species from lower trophic levels (e.g. rodents). They affect the rest of the ecosystem indirectly by enriching the ecosystem with a higher flora diversity around their dens through fertilization by their feces and collection of carcasses (Steneck, 2005, Gharajehdaghipour et al., 2016). Globally the Arctic fox is not considered as an endangered species, however they are categorized as a strictly protected species in the Bern Convention and in Fennoscandia they are regarded as critically endangered (Artsdatabanken, 2015, Eide, 2017). One of the reason for Arctic fox being endangered is disruption of the fluctuation of rodent dynamics which heavily affects the abundance and productivity of the Arctic fox (47%). At low rodent abundance productivity of Arctic fox decreases resulting smaller litter sizes and fewer litter occurrences in the population (Tannerfeldt and Angerbjorn, 1998). Other factors behind endangerment is encroachment of "indigenous" species (e.g. red fox by 20%) which increases competition for resources and increasing intraguild predation which can result chronic stress and deterioration of reproduction (Angerbjorn et al., 2013, Eide, 2017, Boonstra et al., 1998). Other factors endangering the Arctic fox are; small population numbers, inbreeding, human disturbance, hybridizing with farm foxes and diseases (Eide, 2017, Artsdatabanken, 2015, Angerbjorn et al., 2013). There is an association of increased number of Arctic fox juveniles during lemming-years; high food availability during winter results more reproduction and pups born during rodent peak have access to a lot of food during their first year and their survival rate is increased (Strand et al., 1999, Elmhagen et al., 2000, Angerbjorn et al., 1995, Meijer et al., 2013). If rodent abundance severely decline before summer then 90% of the cubs might die of starvation (Meijer et al., 2008, Angerbjorn et al., 2013).

Arctic foxes are adapted to an arctic climate which include the most isolative fur among all mammals (Sillero-Zubiri et al., 2004), their TNZ is estimated to extend between ambient temperature of -45 and -50°C to 26 and 28°C (Klir and Heath, 1992a). Most of the current threats of Arctic fox can be linked to changes in climate and anthropogenic disturbances. IUCN classified the Arctic fox as one of 10 flagship species for climate change (Eide, 2017, Angerbjorn et al., 2013). The reason for choosing Arctic fox as a study species is because they are a native species in Fennoscandia. Because of several threats the Norwegian Arctic fox population is still heavily threatened and would likely go extinct if not for conservation efforts. Arctic foxes are valuable as they are an ecosystem engineer which supporting rodent populations creating a balanced ecosystem (Gharajehdaghipour et al., 2016). By measuring cortisol in each individual fox, we could get an understanding of their physiological

capability to tolerate and react to environmental changes, and help reevaluate conservation measures (Stalder and Kirschbaum, 2012, Yamanashi, 2018). Previous analysis of cortisol levels in Arctic foxes range from an average of ca. 2-5 pg/mg to 1-2 ng/mg, the latter from same populations I used for summer 2014-2015 (Jeannin, 2016, McDonald, 2013).

2.0 Hypotheses

The aim of this study is to investigate Arctic foxes response to ambient temperature and rodent fluctuations in form of fitness and cortisol levels. Measurements of hair cortisol reflects average concentration of serum cortisol of the period the hair strand has grown (Davenport et al., 2006). Measuring stress hormones in individuals can reveal disturbances earlier than demographic methods, because environmental changes often effect physiology of an organism before any changes in the population can be detected (Wikelski and Cooke, 2006, Ellis et al., 2012, Yamanashi, 2018). My hypotheses are:

H01: There is a significant difference of cortisol levels, annual litters, and survival rate in years of abnormalities of temperature and rodent abundance.

Stressors like high temperature and low rodent abundance affects animal health, which is expected to result in increased levels of cortisol, decreased survival and reproductive success.

H02: There is a negative correlation between juvenile cortisol level and survival, as well as between cortisol level and annual litters.

Research shows high stress exposure on juveniles may lower their chance of surviving and reproducing successfully, thus it is expected chronic stress in an early age will lessen an individual's resilience to survive and this will be visual in the populations' breeding success in the future. Bigger litters can lead to competition between siblings for food, I expect wild pups to have a higher cortisol level than captive who experience no food shortage.

H03: Wild born pups, females and juveniles will have a higher cortisol level compared to animals in captivity , males and adults their counterparts.

Pups in captivity are more exposed to anthropogenic disturbances, however because this exposure is consistent individuals should adapt and overtime have a lower stress response compared to wild populations. Females on average have a higher GC concentration because of their different composition of hormones. As long environment don't differ too much from adult foxes' comfort-zone they are expected to acclimate, thus resulting a lower average cortisol compared to juveniles, if any differences.

3.0 Method

3.1 Study Areas and hair sample collection

Most of my samples are of 3-4 months old pups as they are collected during summer for ID-marking, which makes it more non-invasive than if we would recapture adults to avoid unnecessary stress. Captive bred arctic foxes were collected from the Norwegian arctic fox captive breeding station (N=78), and wild born foxes from Dovre (N=69), Vestland (N=45) and Nordland (N=9) in Norway 2014-2018. Sampling was done during July when wild pups were large enough (700-2000 g) to be injected with a microchip (HPT12 PIT-tag, 12.5 mm long, Biomark, Idaho, USA). Captive bred pups were marked by four ear tags with unique color combinations before release (Dalton Rototag®, Dalton ID systems, Nettleved, UK). There were some additional collections of hair (March, August, September and November) when foxes were captured, found dead, or reintroduced to the wild. Resulting in a sampling size of total 427, however half were excluded for technical issues during cortisol analysis leaving 213 individual foxes consisting of adults (N=19) and pups (N=194).

Foxes were caught by tomahawk traps (32 x 10 x12 SLDG DR professional traps) (Landa et al., 2017), baited with cat treat, raw fish or meat. The traps were placed right outside their dens and were checked every 3-4 hours from at least 300 meter distance with binoculars. Captured pups were weighed and hich leg were measured. Ear tissue for DNA and hair from the back of their neck (> 25mg hair) were sampled. Hair samples were stored in a paper envelope, then pups were injected with a PIT-tag between the shoulder blades (Landa et al., 2017) . The pups were masked during the procedure to reduce stress. Conservation of Arctic foxes in Norway include artificial stations with dog food (Landa et al., 2017). The animal trapping and handling was approved by the institutional animal welfare unit, the Norwegian Animal Research Authority (FOTS ID 8946), and the Norwegian Environmental Agency (2013/2412, 2015/5402).

3.2 Hair cortisol extraction

The procedure started with washing off cortisol and dirt from the external part of the hairs with 5 ml methanol (>99%, EMSURE® ACS, ISO, Reag. Ph. Eur. for analysis) in a centrifuge tube (15 ml, 17x118 mm, VWR) shaken 3 minutes with a Vibrax (IKA VXR basic), the methanol was discarded and the procedure repeated two more times (Meyer et al., 2014). The hairs were put to dry on tissue paper, remaining sand or dirt was removed manually with tweezers. The bundle of hairs were held still by tweezers and then cut finely by a nail-scissor above a paper weight boat (Figure 3a). Tweezers transported the hairs from the boat to a reinforced tube (2 ml, VWR) making sure to create a big surface area for the hairs to enhance cortisol extraction. Tubes were marked with the fox id and

weighed (11-30 mg hair), then 1.5ml methanol was added to each tube and placed in vortex with Vibrax for 24 hours for the methanol to extract cortisol from the hair (Meyer et al., 2014).

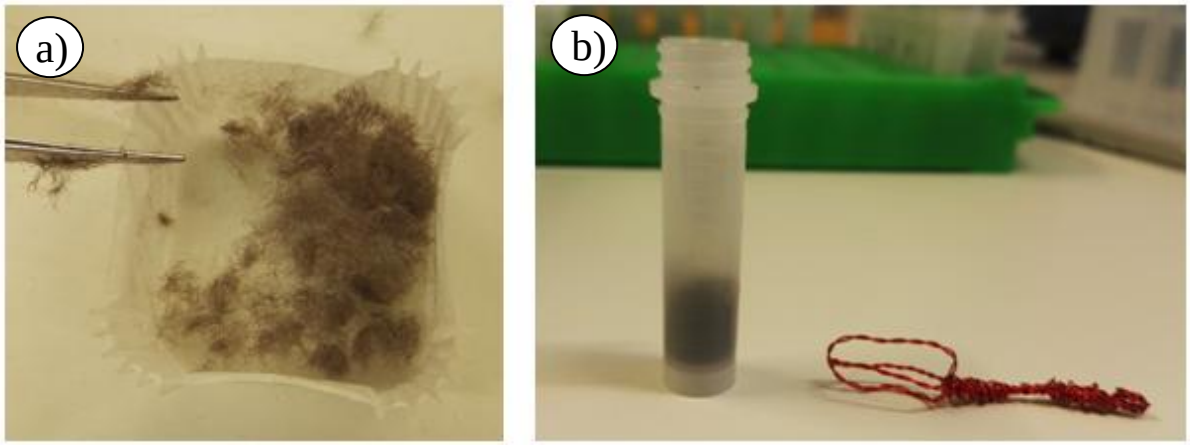

Figure 3. a) Finely cut hair of arctic fox by hand. b) Tube with hair and methanol after 24 hours of vortex and 30 min centrifuge beside a homemade wired wisp to help pressing the hair into compact bundles.

To create a layer of supernatant the tubes were centrifuged (5000 rpm, 30 min). A wisp of copper wire (Figure 3b) was used to press hairs together to make 1.0 ml methanol accessible for transfer. The wisp was slowly submerged, the top of it and bottom of the tube were pressed between the thumb and index finger to press the hair bundle smaller. After about half a minute the wisp was gently removed, waited a few seconds to let floating hairs sink to the bottom before sampling the methanol. Then the methanol was transferred to a new corresponding tube. The wisp was gently hit on a clean paper towel between each sample to remove methanol droplets from the previous tube. The tubes were then placed on a heating plate (38°C) while being evaporated by nitrogen gas (Stuart®; sample concentrator SBHCONC/1 and block heater SBH130D/3) (Meyer et al., 2014). The tubes were checked every 5 to 10 minutes and needles were adjusted so they always were right above the meniscus. After the methanol had evaporated and only cortisol was left, every tube had a few seconds of single tube vortex (VWR International) then assay buffer (250 µl, 1:10 mix) was added (Meyer et al., 2014). If there was debris or small hair strands in the bottom of the tubes, 240 µl of the assay buffer from the dirty tube was moved to a new tube. The tubes were then frozen before analysis (from 1 day to 76 days).

Quality assurance samples were prepared before the start of the experiments. They consisted of a mixture of assay buffer and cortisol from 5 arctic foxes (wild = 3, captive = 1) with two to three replica samples each (total 196.1 mg hair), aliquoted in small volumes so all plates had the exact same extract. These samples were used for experimenting grinding methods, 2 different grinding and cutting combinations were used to increase surface level for cortisol extraction**.** The pool samples were frozen 119 days before analysis started and the last samples were frozen for 148 days. For calculations of cortisol concentration in qualitative samples, 25 mg of hair was used as it has been tested by another master student to be the safest amount to get cortisol readings (Jeannin, 2016).

ELISA is a competitive enzyme immunoassay, it means the assay is run by a competition between an unlabeled analyte (e.g. cortisol) and an enzyme-labeled analyte (tracer) for analyte-specific antiserum binding sites. These form a connection on antibody sites that were cloned from mouse and pre-coated on the plate of wells in which the assay is taking place. After washing away unbound reagents in the wells pNpp Substrate, a solution of p-nitrophenyl phosphate is used to create a color reaction. The strength of the color comes from the amount of tracers bounded to the analyte specific antibody inasmuch as its concentration is known it will be inversely proportional to the concentration of free analyte (Figure 4) (chemical, 2016, ENZO, 2016). With calculations of the OD value and amount of source material the analyte was subtracted from, we can find its concentration in the individual.

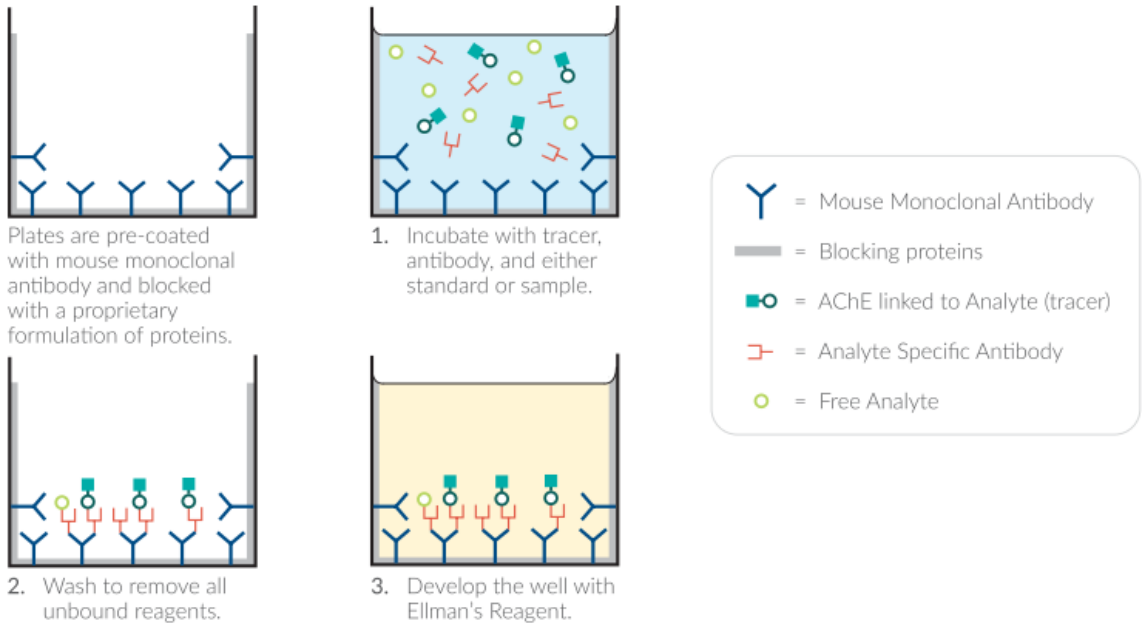

Figure 4. Schematic of an AChE ELISA (chemical, 2016)

As a measure to extract cortisol from hair I used the protocol of Meyer 2014 and used Cortisol ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) to carry out the assay (Meyer et al., 2014, ENZO, 2016). Enzo's ELISA kit do not tell what form of cortisol they measure, so I assume it is values of total cortisol. However, because of technical problems I had to alter the protocols which is addressed in Appendix A. The kits with reagents were stored inside a cool store (4℃). 436 hair samples were divided between 12 plates with 96 wells each. Each plate consisted of duplicates of: blank, total activity (TA), maximum binding (B0), non-specific binding (NSB), standard solutions (standard 1-7), 36-37 hair samples and a quality sample (Appendix B). Few samples were excluded from statistical analysis due to uncertainty of their id. Each plate included hair samples from different categories: foxes born wild or in breeding station, different years and different generations, which were randomly distributed on the plate. When ready to run ELISA, reagents and samples were put to room temperature 30 min before usage (ENZO, 2016). Standard dilutions and assays were made according

to the protocol of the ELISA kit (ENZO, 2016). Assay buffer and wash solution (1:20) were diluted by Qwater (Thermo Scientific™ Barnstead™ Smart2Pure™ Water Purification System 6 UV/UF, PROMO). Samples and reagents were transferred to a 96 well plate. After the last incubation, a stop solution (50ul) was added to every well by a single pipette (ENZO, 2016); plate 7, 10,11 and 12 were measured whiteout stop solution. The absorbance microplate reader Sunrise™ (Tecan Trading AG, Switzerland) and the software Magellan™ (Tecan Trading AG, Switzerland, 2013) measured optical density (OD) of each well at 405 and 620nm, OD value used for calculations are the difference between these wavelengths .

3.3 Data collection

NINA provided data on small rodent cycles from each mountain area used for sampling. Rodent cycles are classified into 4 phases: 1) low (trap index; 0.07+/-0.17), 2) increase (trap index; 2.03+/-1.28), 3) peak (trap index; 8.07+/-8.36), and 4) decline phase (trap index; 0.40+/-0.48), the classification is based on Henden et al. (2009b). The cycles varies between 3-4 years (Meijer et al., 2013, Henden et al., 2009b). The trap-index is between-summers pattern of changes in the number of rodents trapped per 100 trap-nights (Angerbjorn et al., 2013).

To analyze survival a binomial index is used for which 1 = fox detected and 0 = not detected during a 6 month period. Detection measures consist of capture, personal observations or through wildlife cameras, Biomark PIT-tag and DNA extraction from scats. To consider pups surviving their first year they need to be detected after June the year following their birth. I used data of annual wild litters and a list of individual breeders (N=11) from 2014-2018, as an indication of breeding success.

Temperature deviation from normal temperatures (°C) were requested from the Norwegian Meterological Institute (eklima.no). Temperature deviation is compared to historic values measured from 1960-1990 for the month of July, normal temperature calculations for newer weather stations were done by comparing values of neighboring stations. All weather stations used for the analysis are automatic, meaning they record air temperature 2 meters above ground every hour. All data is quality controlled however, all stations have a slight uncertainty (level 2, value not controlled) because the data basis they are generated from are not known well enough . The chosen weather stations are long running stations at the highest altitude within the county from which fox samplings were collected (Table 1).

Table 1. Table of weather stations used for gathering temperature deviation of historic temperatures, extracted from eklima.no (28 Feb. 2020)

Stations

Stnr	Name	Operates from	Operates until	Altitude	Latitude	Longitude	Municipality	County	Region
16610	FOKSTUGU	Jun 1968		973	62,1133	9,2862	Dovre	INNLANDET	SOUTHEASTERN NORWAY
25830	FINSEVATN	Oct 1993		1210	60,5938	7,5270	Ulvik	VESTLAND	WESTERN NORWAY
78800	VARNTRESK	Sep 1999		406	65,8255	14,1942	Hattfjelldal	NORDLAND	NORTHERN NORWAY
97710	ISKORAS II	Sep 2014		591	69,3003	25,3460	Karasjok	TROMS OG FINNMARK	NORTHERN NORWAY

3.4 Calculations and statistical analysis

3.4.1 Calculation of the standard curve

Seven standard samples of known cortisol concentrations were used to fit a 4 parameter logistic (4PL) with log (OD405-OD620) to generate a personalized standard curve for each assay plate to predict cortisol concentration from the samples' optical density (OD). ELISA assays usually have a 4PL like Equation 1, in which the standard curve estimates a, b, c, d and e. Further calculations in Appendix C

$$\log cort = a\,x\,(\log OD)^4 + b\,x\,(\log OD)^3 + c\,x\,(\log OD)^2 + d\,x \log OD + e \qquad \text{Equation 1}$$

In order to judge precision of replicas in each plate and consistency between plates I used percent of coefficient of variation (%CV) of the Cortisol concentration calculated by a 4 parameter logistic curve (Conrad, 2018) (Table 3). ENZO gives 10% as the threshold value between high and low CVs for intra-assay and 15% for inter-assay (Conrad, 2018).

3.4.2 Statistics of stress, survival and litters

The program chosen for statistical analyses was R 4.0.2 (R Core Team, 2018, updated 05.07.2020) performed in RStudio 1.1.456 (© 2009-2018 RStudio, Inc.). Because of expected differences of effect of variables separate models were made for each origin. There seem to be an outliner in the dataset, but when conducting diagnostic plot it is within Cook's distance and thus not removed. Cortisol (pg/mg) is not normally distributed, thus logarithmic values of cortisol were used in all models. Cortisol were transformed back when plotting figures, but SE of log(GC) remained. For analyzing responses behind cortisol levels mixed-effect models from package lme4 were used and I utilized a data-frame of juveniles collected in July and August. To find the best fitted model I used dredge (package: gamm4), REML were set to False during model selection then changed to True when running the fitted model. The saturated model for wild born consisted of the fixed variables; temperature deviation, rodent abundance, fur color and sex of sampling. Including an interaction between temperature deviation, survival and rodent abundance, while plate ID is the random factor. Saturated model for captive born Arctic foxes is based on the one from wild juveniles but sampling month were excluded for lack of variability. The simplest model making biological sense within < 2 ΔAICc was chosen (Appendix D; Table 4). Models of survival were created from bayes generalized linear models with binomial distribution (package: arm). Full model of captive juveniles survival consisted of logarithmic cortisol values interacting with plate, rodent abundance, temperature deviation, sex and fur color. Then I manually removed non-significant terms stepwise and used model.sel (package: MuMIn) to compare Akaike's Information Criterion (AIC) of all models (Appendix D; Table 5). A model with just estimates of mean Survival was included in the selection to see if the other models have any explanatory power. Lowest ΔAIC and/or models with lowest degrees of freedom within 2 ΔAIC were chosen. Models predicting annual litter occurrence were modeled after generalized linear models with poisson distribution (package: lme4 and car). I performed a

dispersontest (package: AER) and found neither over or under dispersion, however diagnostic plot showed data is negatively skewed. Then I ran Pearson's product moment coefficient to check for correlation between wild litter and cortisol level in wild summer pups and adults. When there was a correlation I did model selection to further investigate. Full model was made for wild pups and it consisted of logarithmic cortisol interacting with plate, rodent abundance, temperature deviation, sex and fur color. Like for models of survival a model selection was utilized by AIC (Appendix D; Table 6).

Pearson correlation tests was utilized between two numerical variables and when there are a linear relationship between them. Judging the strength of the correlation as follow; strong: ±0.80 >, moderate: ±0.80 > ± 0.40, weak: ± 0.40 >. Chi-square test was used when correlation involved at least one categorical variable. For the last hypothesis two-tailed t-test was used to compare cortisol levels in wild pups vs. captive pups, adults vs. pups, females vs. males, and two negative binomial models (package: MASS) were made for box-plotting of cortisol. Relationships and patterns were read out of the models summary. Graphics were done in RStudio (packages: reshape2, ggplot2 and ggeffect).

4.0 Results

There was enough fur to analyze at least one sample from each of the 427 Arctic foxes captured between 2014 and 2018. 36-37 foxes were analyzed on each assay plate. Chi-square correlation test was performed on the remaining cortisol samples with their representative plates to see if there was a relationship between cortisol values and plate number. The test showed a significant correlation between plates and cortisol levels (χ^2= 270.03, df = 225, p = 0.021), this was accounted for in models by including log(GC) interacting with plate ID. When examining how cortisol changes through 2014 - 2018 there is also a correlation (χ^2= 214.89, df = 180, p = 0.039) of which 2015 (n= 23) Arctic foxes had an overall higher cortisol level (4.35±0.39 pg/mg) compared to other years (2014; 2.12±0.23, 2016; 2.44±0.32, 2017; 2.57±0.19, 2018; 1.97±0.20 pg/mg). Range of cortisol in wild juveniles; 1.0 - 7.7 pg/mg, median = 2.1. Cortisol of captive juveniles; 1.1 - 17.4, median = 1.9.

4.1 Stress and environmental factors

Wild and captive pups were analyzed separately for relationships between level of stress, temperature deviations and rodent abundance; level 1 - low phase (N = 15), level 2 - increase phase (N = 14), level 3 - peak phase (N = 94), level 4 - decline phase (N = 64).

Wild born pups (N=109) cortisol level has strong relationships with all rodent phases with a focus on peak and declining abundance (All levels; p< 0.001, Level1; SE=0.17, Level2; SE=0.14, Level3; SE=0.10, Level4; SE=0.11, Figure 5a). Temperature deviance (°C) for historic July temperatures has a significant effect (p< 0.001) on cortisol in wild born. There is a weak negative correlation in wild cubs

(df = 107, p< 0.001, cor = -0.38) by a factor of -0.11 ± 0.02 pg/mg pr temperature unit (Figure 5a). Cortisol levels in captive born pups (N=78) have a different relationship to rodent abundance than wild born (Level1; p< 0.001,SE = 0.15, Level2; p=0.05, SE = 0.21 Level3; p< 0.01, SE = 0.17, Level4; p<0.1, SE = 0.26, Figure 5b). Juveniles in a breeding station show highest levels of cortisol during local rodent phase 1 (low), followed by phase 4, 3 and 2. Cortisol plates explain 32% and 12.8 % of variance that is not explained by the fixed effects in wild and captive pups respectively (Appendix D; Table 5).

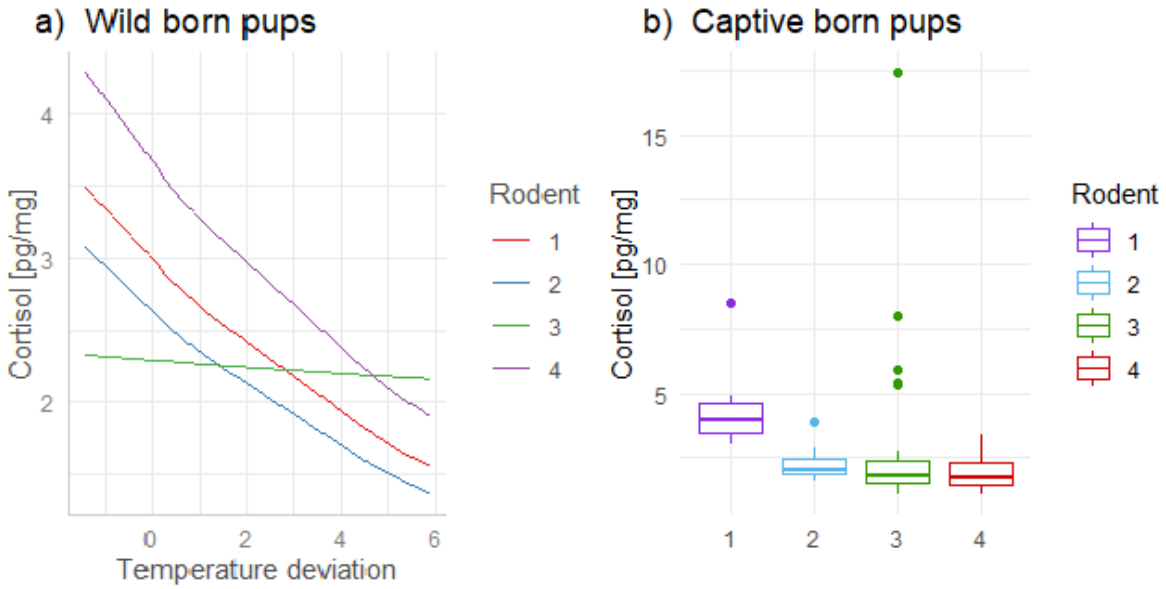

Figure 5. Best fit models predicting cortisol [pg/mg] levels from Arctic fox pups, hair samples collected July and early August. a) Wild born juveniles cortisol is best predicted by temperature deviations of historic July temperatures, rodent abundance, an interaction between rodent phase and temperature deviation and cortisol plate as randome effect. Local rodent cycle (1-4) level 1: low (N=5), level 2: rising (N=7), level 3: peak (N=59), level 4: declining (N=39). b) Captive born juveniles cortisol is best predicted by rodent abundance and cortisol plate as randome effect. Local rodent cycle (1-4) level 1: low (N=10), level 2: rising (N=7), level 3: peak (N=35), level 4: declining (N=26).

4.2 Survival

Only 3 of 109 wild pups have been observed after their first year, thus lack of variation prevent me from performing survival analysis of wild born Arctic foxes. Cortisol does not have a significant effect on survival of captive born pups (χ^2 = 34.48, df = 32, p = 0.35), neither does temperature deviation(χ^2 = 2.72, df = 4, p = 0.61) . Model selection of variables and interactions predicting survival of captive bred foxes favor solo variables of temperature deviation and cortisol (Appendix D; Table 6) . However, these have a < 2 ΔAIC after a model estimating mean survival, meaning they are equally likely thus have no significant effect on survival (Figure 6).

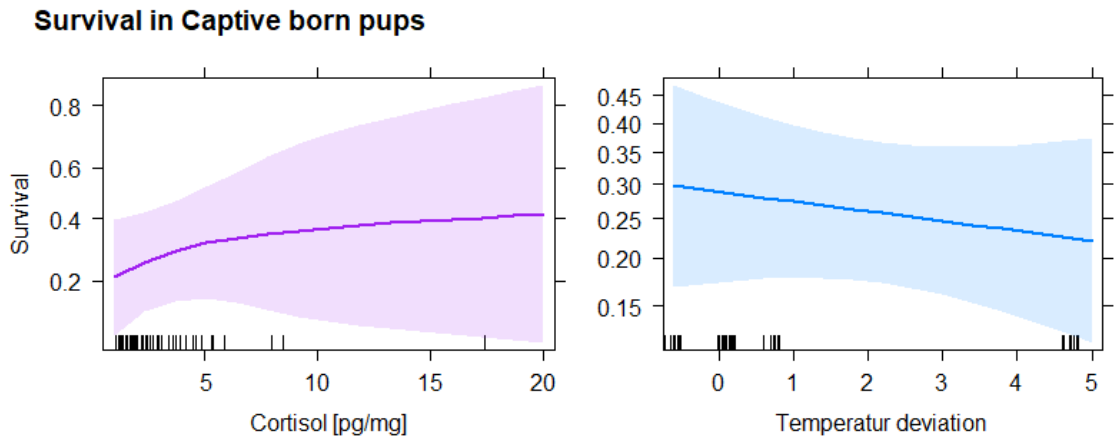

Figure 6. Survival of captive born Arctic fox juveniles is best described by temperature deviation (°C) and cortisol [pg/mg] according to model selection. They are in order from left to right after strongest AIC weight, but they are not statistically significant. Cortisol [pg/mg] levels from hair was sampled July and early August.

4.3 Stress and annual litters

Individual level of stress in captive foxes is not correlated with wild annual litters (df = 76, p = 0.1, r = -0.19) whereas wild foxes have a negative correlation (df = 107, p < 0.05, r = -0.21). Increased litters explains 4.8% of cortisol levels in wild pups (N = 109) and 7.6% in wild adults (N = 19).

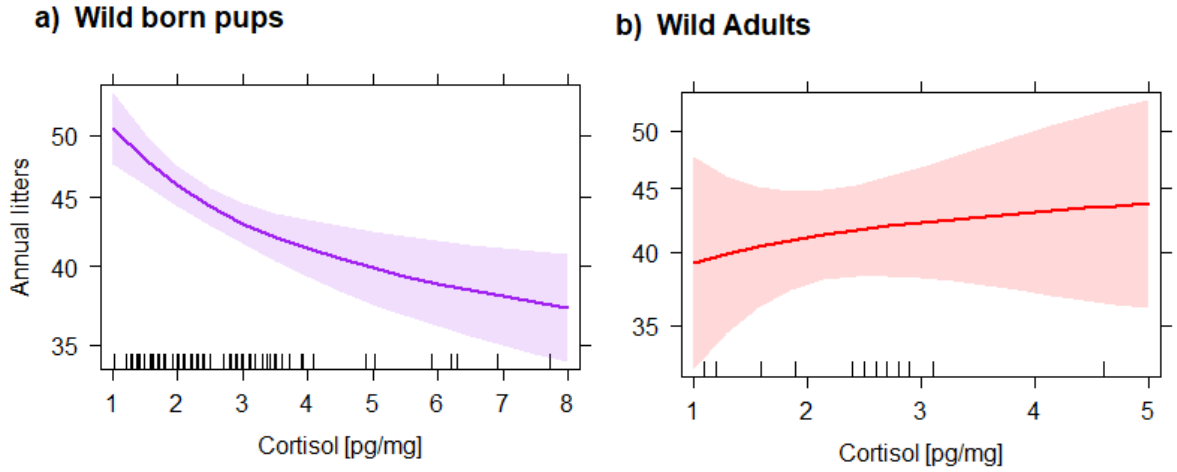

Figure 7. Cortisol [pg/mg] of Arctic fox cubs in relation to litters counted in Norway the year of hair collection. Cortisol values collected from July and early August. a) Wild cubs (N = 109): significant correlation, b) Wild adults (N = 19): no correlation.

However, correlation analysis show cortisol levels in adults do not correlate with annual wild litter occurrences (df = 17, p = 0.29, r = 0.26). Eleven Arctic foxes could be used to explore relationship between stress and individual reproductive success, reproductive success was calculated from average cubs produced each time they bred. There were no correlations (df = 9, p = 0.09, r = 0.54). Of these Eleven three had hair sampled when they were pups, their stress level as cubs were not correlated with their reproductive success (df = 1, p = 0.08, r = 0.99).

Best fitted model predicting annual litters based on data collection from wild juveniles consists of rodent abundance and temperature deviation (Appendix D; Table 6). This and lack of correlation illustrating environmental factors have a greater effect on breeding success than stress. Wild breeding success had a positive correlation to rodent abundance ($\chi^2 = 329.11$, df = 9, $p < 0.001$) and July temperature deviations (df = 210, $p < 0.001$, r = 0.72).

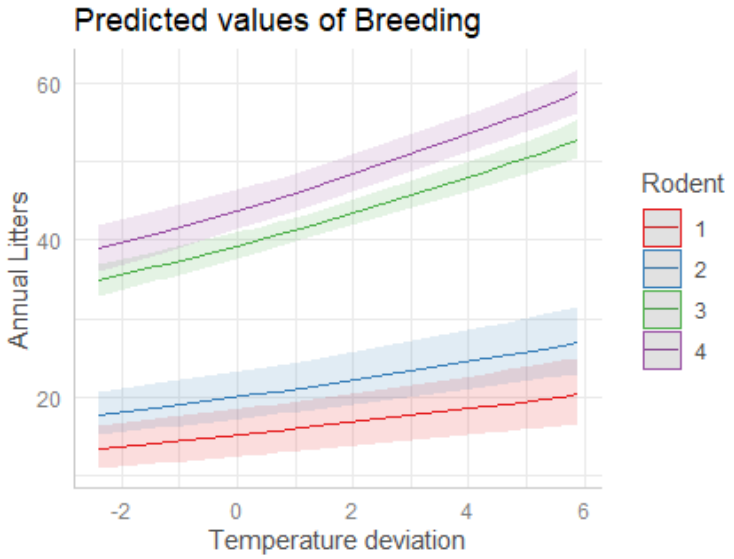

Figure 8. Predicted plot of relationship between observed annual wild litters, temperature deviation of July and summer rodent phases in the wild (Level 1 - low, level 2 - increasing, level 3 - peak, level 4 - decreasing).

4.4 Characteristics most exposed to high levels of stress

T-tests show no significant difference of mean cortisol level between: captive and wild pups (p=0.85 SE=0.093, Figure 9a), male and female(p=0.7 SE=0.093, Figure 9b), pup and adults (p=0.66 SE=0.17, Figure 9c, Table 2).

Table 2. Average cortisol concentration found in Arctic fox hair of cubs born in captivity and in the wild during summer, average cortisol of all females and males, and average cortisol of all juveniles and adults

	Average GC [pg/mg] in hair
Captive bred pups (N=78)	2.58 ± 0.09
Wild bred pups (N=109)	2.52 ± 0.09
Female (N=102)	2.45 ± 0.9
Male (N=84)	2.67 ± 0.9
Pups (N=193)	2.54 ± 0.17
Adults (N=19)	2.08 ± 0.18

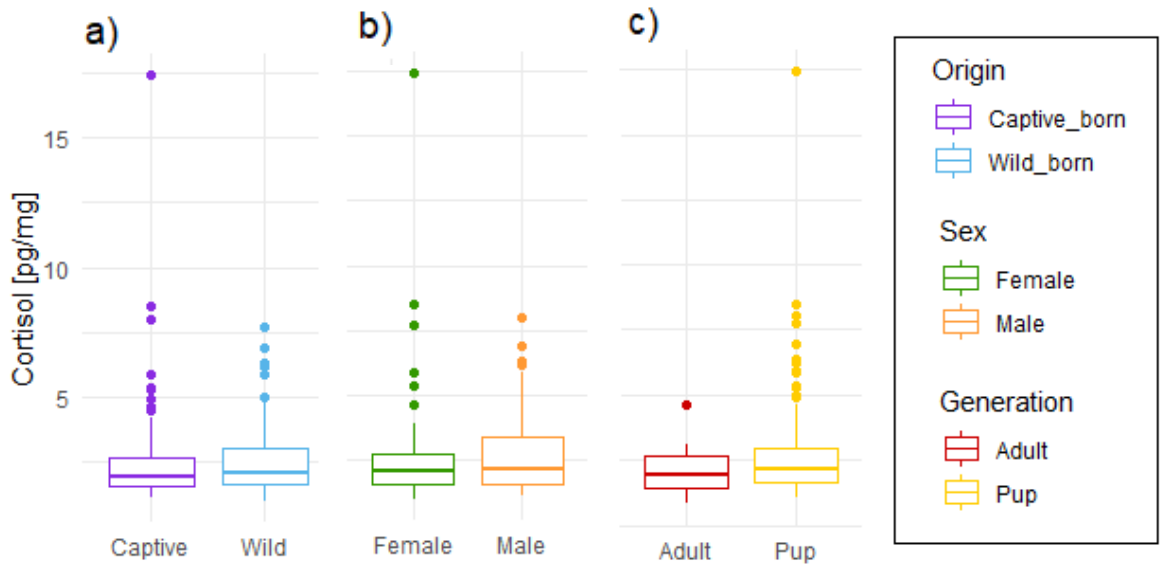

Figure 9. Pattern of cortisol level between; a) Summer cortisol level between captive and wild born arctic fox pups. b) Cortisol level between female and male arctic fox adults and pups. c) Cortisol level between adult and juvenile.

4.5 Intra- and Inter-plate %CV

Result from Intra-Assay show only plate 6 is under the threshold of good replication level between samples (Table 3). The CV of the other plates don't differ that much, except plate 1. When removing plate 1 and updating Inter-Assay % CV it resulted 23.0%CV. Thus samples from plate 1 are still included for analysis.

Table 3. Intra CV for each plate analyzed and inter plate CV. Calculations in Appendix C.

Plate ID	Intra-Assay %CV
1	19.5
6	9.4
7	10.5
10	12.9
11	10.9
12	10.2
Inter-Assay %CV	20.8

5.0 Discussion

5.1 Cortisol levels in Arctic foxes

Arctic foxes usually have ca. 2-5 pg/mg cortisol in hair (n=53, McDonald (2013)) my results are on average within this range. It is worth noting that McDonald used radioimmunoassay instead of ELISA, so some differences are to be expected. My collection of Arctic foxes might be biased towards individuals with an assertive personality that are more curious and explore the cages more thoroughly.

Usually assertive personalities have a low GC secretion (Blas et al., 2007, Cockrem, 2013). Average cortisol values of different origins, generation and sex are not significantly different (Figure 12, Table 3). This is against my expectations of higher cortisol levels in wild pups, females and juveniles. This could imply one characteristic is not more exposed to stress than the others and cortisol levels do not differ with age. The last implications justify the relevance of measuring stress in pups to estimate level of stress in the rest of the population. Even if my cortisol values are not precise, the large amount of samples gives a good impression of patterns, when comparing samples within the same plate there is a consistent pattern of cortisol concentrations between years and origin (Appendix E). To compare with another arctic carnivore, polar bear (*Ursus maritimus*) has hair cortisol range of 0.2 - 5.8 pg/mg (n = 378, Bechshoft et al. (2015)), mine range of 1.0 - 7.7 pg/mg hair in wild Arctic fox juveniles do not seem so unlikely.

Cortisol concentrations are highly individual dependent, extreme low and high values do occur naturally resulting in high standard deviations because of genetic differences, pre- and post-natal and adult experiences (Cockrem, 2013). Which can make it challenging to receive a significant correlation with other variables. However, wild cubs have a stable level of stress during different temperatures when rodent abundance is high (Figure 5); this is also reflected by its low SE. Rodent abundance can be an indicator of net primary productivity of an ecosystem (Hersteinsson et al., 2009) and are essential for Arctic foxes as a food source (Sapolsky, 1985). Foxes in captivity are not dependent on rodent abundance thus any correlations between captive bred foxes and rodent cycle might be the result of correlation or effect with a common environmental factor. The significant link between wild born pups cortisol and rodents implies rodent abundance has a stronger predictive power over stress response in wild born pups than in captive. This makes sense as wild foxes have rodents as their main food source, however this effect might be greater in populations with no supplemental feeding. Seeing a stable cortisol level in wild cubs during abundant years suggest peak rodent phase might provide enough to feed big litters or lessen pressure of other stressors. Although measurements of captive born pups show highest cortisol levels during phase 1, wild born pups have this effect for phase 4 which is a declining phase after a peak phase. Rodent abundance tends to crash after a peak and sudden changes can inflict stress. When foxes are used to overflowing abundance and this starts to decrease they might get more stressed than during other phases. Foxes live on average 3-5 years, meaning they usually don't live long enough to learn the rodent cycle, thus they can perceive the environment as unpredictable which creates psychological stress. This might be illustrated in figure 5a in which cortisol peaks at declining phase then cortisol gradually decreases at following phases. Starvation can also causes a stress response by forcing adult Arctic foxes to travel longer distances to find food and might have to compete with red foxes. When male baboons in the Serengeti are forced to forage over a longer distance their GC increased (Sapolsky, 1985). A high litter count under phase 4 can be a result

of parents previously experiencing high rodent abundance, and lowest litter count during phase 1 can be a result of low rodent abundance the year prior and a peak low current year.

5.2 Stress and environmental factors predicting breeding

Kalliokoskiet et al. (2019) studied the legitimacy of the link between stress and hair GC, in his research he concluded the link between them is strong only when the stressor is present when collecting the hair and not after it has passed. In my study rodent phases are calculated from rodents captured during summers on the mountain area and my temperatures is from July. These stressors are present during collecting period. Arctic foxes molt twice a year, thus hair samples of adults consist of average cortisol concentrations the past few months. Meaning their stress level is closer related to summer rodent abundance compared to winter abundance. As previously mentioned stress can increase the risk of becoming less fertile (Sapolsky, 1985). I found no correlation between level of stress in adults and reproductive success (neither on an individual level or as a population) (Figure 8b). Figure 8a illustrates a negative correlation between cortisol in wild pups and annual litter, it can be seen as a continuation of rodent phases in figure 7. Low level of stress when there is more litters could be a reflection of good environmental conditions and there are no major competition between cubs (Tannerfeldt and Angerbjorn, 1998). Higher levels of stress in wild pups at low reproductive success could biologically be explained by maternal imprinting and/or increased competition within litter for food.

Annual wild litters have a correlation with temperature deviations (Figure 7). This implies that a < 5°C deviation can have a positive effect on Arctic fox fitness, ergo a threshold of maximum rate of the performance curve has not been surpassed (Figure 2). Hersteinsson et al. (2009) discovered warm summers improve life of Arctic fox populations on Iceland. It is suggested that climate conditions which increase net primary productivity during their growth phase affects pups final size by influencing energy metabolism for maintenance and food availability, increased size and strength increases survival (Hersteinsson et al., 2009). Therefore can warmer summers in Norway positively affect population growth of Arctic fox. This is also shown of increased sample size of pups during high rodent abundance. However, there are cases of high abundance of litters during low rodent abundance, e.g. in 2015, this makes sense when rodents are not their only food source, e.g. food stations (Hof et al., 2012). Effect of low rodent abundance in 2015 might have a link with increased cortisol in juveniles (Appendix E). It is possible high rodent abundance during winter can stimulate the vixens to carry more litters because the environmental conditions were favorable before and/or during pregnancy. Although almost 90% of the cubs die of starvation during the summer (Meijer et al., 2008). To examine this I could compare rodent abundance the year prior.

5.3 Heat stress in Arctic foxes

In some cases animals can acclimate to temperatures and develop less sensitivity for a reoccurring stressor, e.g. anthropogenic activities. Thus it makes sense to use temperature deviations rather than average temperatures to judge the effect of a changing climate. By using temperature deviations you know to what degree changes occur. At temperatures above an individual's comfort zone, physiological responses like sweating and panting occur. This critical temperature depends on food and water homeostasis which is compromised during drought (Mitchell et al., 2018). My results show that there is a negative correlation with higher temperatures and cortisol, this is opposite of what I expected (Figure 5). Reason for increased cortisol levels during colder summers might be a reflection of how the state of the ecosystem indirectly affects the arctic fox. Furthermore, warm summers are becoming more common in Norway, my dataset include two extreme warm summers (2014, 2018) and one summer (2015) with less intense < 0℃ temperature deviations. 2015 is also one of the years with the least samples (N=23) resulting in a smaller sample size of foxes in colder years, this can result in a false positive. A reason why cortisol decreases with increased heat might be because the foxes become more immobile, to escape long or intensive heat they stay inside their dens. Arctic foxes do not hunt when the sun is at its strongest, therefore an overall increase of temperature could force foxes to stay inside their den for a longer period. Arctic foxes are most active at dusk and dawn in this timeframe they might hunt enough to prevent starving midday. Thus preventing a rise of cortisol secretion. I have yet to find an article covering behavioral change of Arctic foxes at high summer temperatures and most studies on thermoregulation in Arctic foxes are about how they tolerate extreme cold during winter. However, Klir and Heath (1992a) measured resting metabolic rate and evaporative water loss in two adult male arctic foxes, they concluded that upper critical temperature is probably between 26 and 28 ℃ ambient temperature. The highest average monthly temperature among my study sites was 17.4 ℃ at Saltfjellet (520 moh) in 2014 and the closest long-running weather station, Varntresk (406 moh) (Table 1), had at the time a temperature deviation of 5.9 ℃ from normal. These ambient temperatures are within the Arctic fox's presumed TNZ, however, these air temperatures are underestimating the intensity of sun radiation at high altitudes (Mitchell et al., 2018). Soon after the previous article Klir and Heath (1992b) performed a study on external thermal stress from -25 to 33 ℃ in six adult arctic foxes in captivity in the USA. They reasoned Arctic foxes experiencing temperatures down to -50 ℃ may experience heat stress to summer temperatures reaching 20 ℃, but they reached no conclusions on that matter. My research shows no link of increased stress at ambient temperatures above normal. In theory cortisol affinity for CBG decreases at higher temperatures resulting in more free cortisol in plasma at low total cortisol (Breuner et al., 2013), making it difficult to see a strong link with total cortisol which can explain lack of significant results. Unless cortisol in hair is predominantly of free from, then a lack of correlation can result from high inter assay %CV.

5.4 Survival linked to level of stress in Arctic foxes?

Rodents are a major component determining survival and reproduction in arctic foxes (Angerbjorn et al., 2013). Sadly not enough wild cubs have been observed one year after their birth to result a comparable sample size between survived and presumed dead. I expect lower survival of wild pups during periods of low rodent abundance because restricted food source. Moreover, increased time hunting might be needed when rodents are scarce, this can create a tradeoff between food and protecting cubs, parents spending more time away from the den expose their litter to a higher risk of intraguild predation by wolverines, birds of prey and red foxes (Erlandsson et al., 2017, Tannerfeldt et al., 1994). Survival is expected to be strongest in captive bred foxes as they are protected in enclosures their first 6-7 months then released to the wild in January-February. When running model selection for survival of captive pups best fit models include either temperature deviation or cortisol (Figure 8, Appendix D; Table 5), however there is no correlation with survival. A zero in their confidence interval further proves they are not supported statistically. Reason for rodent abundance to not be better fitted for survival of captive pups could be because of their high standard deviation. This could be a result of dividing and releasing juveniles to different mountain areas with different rodent phases during winter, my collection of biotic data is from the environment they were born into. Winter feeding has the most impact on survival, but rodents are counted in the summer. To account for this I could compare rodent abundance the year prior instead.

Cortisol seems to not have a significant link to fitness. There is a lot of discussion if hair can record past stress levels, and thus simulate long-term stress. Either cortisol does not reflect stress level or my findings show Norwegian Arctic foxes are not chronically stressed or fitness is not negatively affected by it. Previous experiments found differences in susceptibility to glucocorticoids between species. Foxes are closely related to species tested and categorized as GC-resistant, so I assume Arctic foxes are less effected by GC (Claman, 1972). Hamsters, mice and rats have been tested and categorized as GC-sensitive, thus more prone to being affected by environmental stress (Claman, 1972). Lemmings are highly sensitive to climate change (Schmidt et al., 2012).Thus environmental stress will have the biggest impact on Arctic fox indirectly.

Further studies should focus on clarifying the mechanisms behind incorporating cortisol into hair strands and of what form they are. There is also a need to continue researching properties of free and total CBG to understanding their roll in stress-response. If these things are better understood then it is easier to draw clearer conclusions from measurements of hair cortisol. To account for microclimate a study can collect temperature data from satellite pictures or utilize other methods to receive precise or local temperatures. Collecting data on how much time foxes use outside their dens at different years would be interesting to see if they do spend more time in their dens during warm summers which could possibly be behavioral adaptation to reduce the release of free cortisol in their circulation. In

order to get a clearer view of the relationship between ambient temperature and litter size a time frame beyond 5 years is needed as a normal lemming cycle is 3-4 years.

Conclusion

I found no significant link of cortisol affecting fitness or welfare in Arctic foxes. In this case study hair cortisol in juvenile Norwegian Arctic foxes from the breeding station do not correlate with abnormal temperatures nor survival. Hair cortisol concentrations in wild juvenile Norwegian Arctic foxes however, correlate with rodent abundance and have a weak negative correlation with ambient temperature deviations. Pups of different origins are affected differently by rodent phases regarding cortisol level in hair: wild juveniles have a stable cortisol level during peak phase and highest cortisol concentration during declining phase. Captive bred juveniles show highest cortisol levels during the low phase of rodent abundance. As foxes in the breeding station are not dependent on rodents for nutrition, this link is believed to reflect effects of another variable. I found no correlation of an adult's cortisol level and their reproductive success, nor did I find any correlation with annual population reproductively.

My findings show a significant positive link between temperature deviation of 5°C above historic temperatures and reproduction of Arctic fox. Implying increased temperature improves productivity of arctic/alpine habitat which can support more litters. It is expected alpine habitat will change with prolonged exposure of increased temperature, and we cannot be sure how Arctic fox will handle this. Right now they seem to gain from increased net production of the ecosystem. I conclude that Arctic foxes are most likely GC-resistant and can handle stress well. Fitness and welfare are more likely to be predicted by rodent abundance and temperature than.

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Appendix A

Due to the complications with my protocol I cannot exclude the possibility of my cortisol values not mirroring natural occurrences. However, when including cortisol and plate ID interaction in saturated models the interaction was never close to be a best fit.

Method alterations

The method for grinding hair with three 3mm steal beads did not work, after several rounds and a total time of 7 and 10 min in the grinder the hairs bundled together instead of turning to powder, 13 minutes centrifuge did not help much either. This is likely because of the fine properties of Arctic fox hair. I added some methanol to the tubes to see if the grinding would be more efficient with the hair floating (Jeannin, 2016), this was not the case. I also cut the hairs between grinding rounds, this resulted loss of hair and hair bundles were still formed during mincing. Previous research shows that grinding hair does not extract more cortisol than finely cutting the hairs (Slominski et al., 2015). The usage of steal beads for grinding inside a plastic tube could result in releasing pieces of plastic preventing exposure of hair particles to methanol, chemical degradation of hair protein and/or steroids might also occur during the milling process, meaning cutting hair may be safer than grinding (Slominski et al., 2015). Due to limited hair samples and time, the rest of the protocol was not performed to see the domino effect of cutting hair before performing the experiments. With the hairs finely cut they created a greater volume absorbing methanol making it difficult to get 1 ml of supernatant. Bigger tubes and more methanol would help, however the dilution ratio would change. A handmade wisp was made out of copper wire to press methanol out of the hair. I chose a red coated copper wire to increase the probability of observing if part of the outer coat sheds. This decreased the amount of debris in the supernatant, however there were some contaminations. To combat this I checked each tube after evaporating methanol, if contaminated I added assay buffer 250 µl and transferred 240 µl of it to a new tube avoiding the debris (checked if this affected results as I went). Chi-square test conclude there are no correlation between cortisol value and volume of assay buffer (χ^2= 59.805, df = 45, p = 0.06879). This does not rule out the possibility of micro particles in the wells, hopefully they remain at the bottom and not in which OD value is measured resulting incorrect OD measurements of cortisol. In order to secure the cortisol measurements only plates with no problems were used for analyses, a correlation was found between plate and cortisol (χ^2 = 270.03, df = 225, p = 0.02134). Then a qualitative value was calculated by cortisol value from the plates pool and mean of all samples approved for analysis. ENZO gives 10% as the threshold value between high and low CVs for intra-assay and 15% for inter-assay (Conrad, 2018). Most of remaining plates have an ok CV, CV for plate 1 surpasses this threshold (Table 4) but inter-assay increases when excluding plate 1. So I chose to keep plate 1 to increase the sample size.

During reading of plates 8-9 the last half of the plates had cortisol below detection levels, OD reading of the remaining plates 7, 10,11 and 12 were executed before adding the stop solution every 5 minutes for 20 minutes, then the stop solution was added and measured OD according to protocol. The next 10-15 minutes I continued to do readings every 5 minutes to see if there are any changes post stop solution. Then the reading with the most samples above the reading level was chosen (plate 7; 20min after ended incubation, plate 10; 0min after ended incubation, plate 11; 10min after last incubation, plate 12; 0min after last incubation). It takes 10 minutes to carefully apply the stop solution from standard solutions to pool samples at the end. Though the readings used ranged from an extra incubation period of 0-20 minutes this was consistent for every well within each plate. Cortisol values are calculated from standard samples within each plate, a longer incubation period does not change the relations within the plate and by using qualitative cortisol values to normalize samples on the different plate, the possible effect of 20 min longer incubation is diminished. I believe this method is better as it reduces changes for cross-contaminations and in-equal incubation period within plates when adding stop solution. I defined a categorical value describing combination of methods to every cortisol sample and ran a Chi-square test ($\chi^2 = 102.43$, df = 90, p = 0.1746). No correlation between cortisol levels from different methods.

Previous master student concluded >25mg Arctic fox hair is needed to extract enough cortisol for the plate reader to read.However my alterations of the method made it possible to extract enough cortisol down to 11.6 mg hair. To include more individuals for the analysis I included hair samples < 25 mg. By running a Pearson's product-moment correlation test I see there are negative correlation ($p < 0.005$, df = 210, r = -0.19) between amount of hair and uncorrected cortisol extracted (aka. cortisol values not normalized). Cortisol concentrations increased as hair weight decreased, big amounts of hair created problems during my experiments as they occupied more space in the 2 ml tube forcing me to manually press down the hair to access methanol with cortisol extraction. During my test grinding I had one sample made of 14.4mg hair which was minced well. If I had the time to run a test assay with < 20mg hair samples to see their efficiency of extraction cortisol all issues linked to hair samples could have been avoided.

Enzyme-linked immunosorbent assay

Enzo's ELISA kit has a cross-reactivity of 122.35% with prednisolone and 27.68% with corticosterone (ENZO, 2016). These like cortisol are common glucocorticoids and connected to stress. The reason I measure cortisol is to get an estimate of stress, thus possible cross-reactivity will not affect my results (Sigma-Aldrich, 2020). Enzo's cortisol ELISA kit binds to total glucocorticoids, as previously mentioned during introduction it might only be free cortisol causing stress reactions (Breuner et al., 2013). Foxes with high cortisol concentrations might have predominantly CBG resulting little effect of stress. More research is needed to support usage of total GC as an estimation of stress. An experiment comparing different commercial ELISA assays for resulting similar values of corticosterone found Enzo ELISA kits to have low values compared to Arbor Assays kits and DRG-4164 (Rod et al., 2017), it would be interesting to see a similar experiment repeated with analyzing different steroid compounds e.g. cortisol and see if Enzo ELISA kits have a trend to yield lower values compared to other kits. This is important for comparing results and defining the true concentration of steroids in samples. However, ELISA kits can still determine relative differences between samples (Rod et al., 2017).

Figur 10. OD (405-620nm) reading of plate 6. BL: blank samples, TA: total activity , B0:maximum binding, NSB: non-specific binding, ST1-7: standard samples, SM1-36: hair samples, POOL: qualitative samples.

Appendix C

3.4.1 Cortisol calculation

Raw data were extracted from Magella to an excel sheet. Calculated average OD405-OD620 from duplications. For each sample and controls following calculations was done;

Average net OD = OD405-OD620 - NSB

Log OD = log (Average net OD)

log [cortisol] = log of cortisol consentration predicted by standard curve

[cortisol] pg/mL = log10[cortisol] multiplied with dilution ratio (ratio=1 in this case)

$$[\boldsymbol{Hair\ cortisol}]\ \frac{\boldsymbol{pg}}{\boldsymbol{mg}} = [cortisol]\ pg/mL * 250\ \mu l\ (assay\ buffer\ /\ 1000\ /\ Hair\ weight\ (mg)$$

(Samples that needed transfer to a new tube were calculated with 240µl instead)

Data were normalized due to batch effects; the samples were analyzed in 12 separate plates, completed on different days during one month time, while the method behind some plates had to be adapted to changes. To correct for this I used pool values from respective plates;

$$\boldsymbol{Qualitative\ hair\ cortisol\ relative\ to\ pool} = [Hair\ cortisol]\frac{\text{pg}}{\text{mg}} mean\ /\ Hair\ cortisol\ pool * Total\ mean\ accross\ all\ plates$$

3.4.2 Intra and Inter assay CV

Intra-plate CV is an average of CV values from each sample and quality controls. This illustrates consistency within a plate.

$$\boldsymbol{CV} = Sd\ of\ [cortisol]pg/mL3\ /\ Average\ of\ [cortisol]pg/mL2 * 100$$

Interplate CV calculated from Quality control on every plate, it shows the consistency between plates.

Appendix D

Table 4. Top 4 mixed effect models from model selection through fitting likelihood of summer Juvenile Arctic fox. GC - cortisol pg/mg, R - rodent abundance, Td - July temperature deviance, F - fur color, S - survival of first year. Best fitted model is marked.

		Models of Stress	AIC	ΔAIC	weight
Stress	Wild Pups	**Log(GC) ~ R + Td + R:Td**	87.57	0.00	0.184
		Log(GC) ~ F + R + Td + R:Td	88.70	1.13	0.104
		Log(GC) ~ R + S + Td + R:Td	89.04	1.47	0.088
		Log(GC) ~ R + S + Td + R:S + R:Td + S:Td	89.04	1.47	0.088
	Captive Pups	Log(GC) ~ R + Td	101.9	0.00	0.122
		Log(GC) ~ R + Td + R:Td	101.9	0.00	0.122
		Log(GC) ~ F + R + T + R:Td	103.2	1.23	0.066
		Log(GC) ~ R	103.7	1.75	0.051

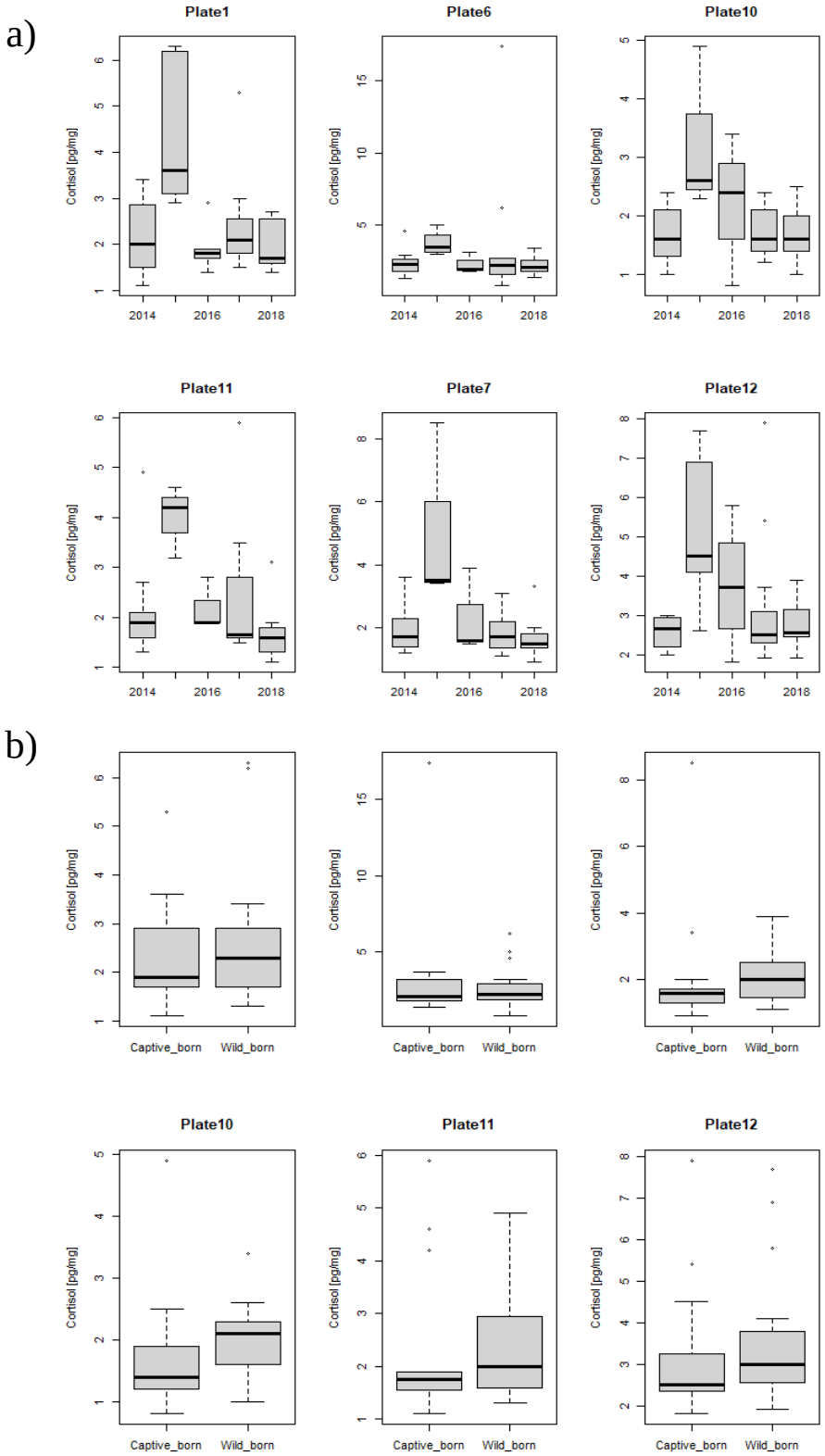
Tabell 5. Top 4 binomial generalized linear models from model selection through fitting likelihood of Juvenile Arctic foxes from the breeding station. GC - cortisol pg/mg, Td - July temperature deviance. Best fitted model is marked.

		Models of Survival	AIC	ΔAIC	weight
Survival	Captive Pups	**Survival ~ 1**	90.9	0.00	0.356
		Survival ~ Td	92.4	1.54	0.199
		Survival ~ Log(GC)	92.4	1.56	0.197
		Survival ~ Log(GC) + Td	94.4	3.50	0.075

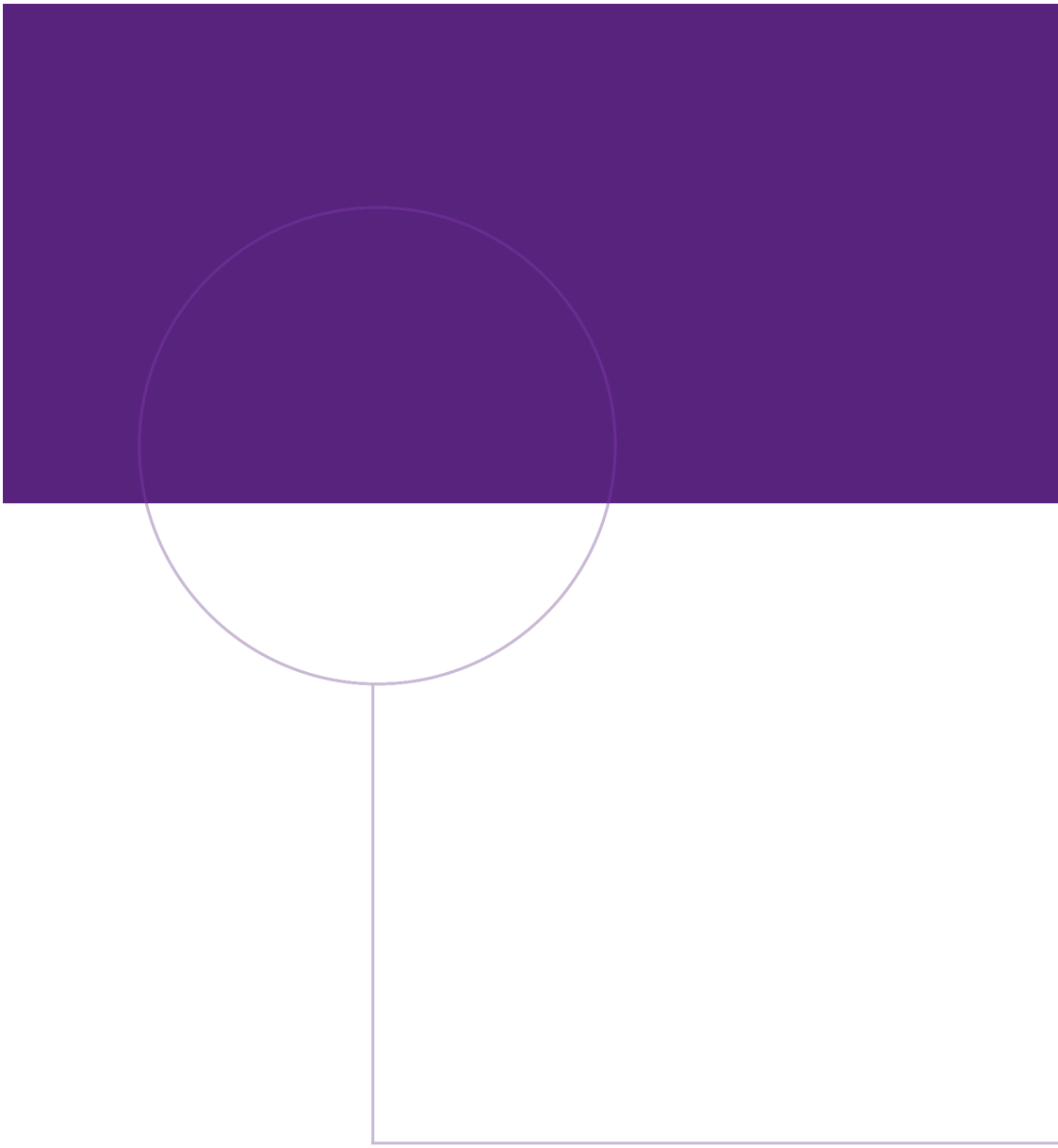
Tabell 6. Top 4 poisson distributed generalized linear models from model selection through fitting likelihood of summer Juvenile Arctic fox. GC - cortisol pg/mg, R - rodent abundance, Td - July temperature deviance, F - fur color, S - survival of first year. Best fitted model is marked.

		Models of Wild Litters	AIC	ΔAIC	weight
Litters	Wild Pups	**Litters ~ R + Td**	621.6	0.00	0.387
		Litters ~ R + Td + R:Td	623.0	1.42	0.190
		Litters ~ F + log(GC) + R + S + Td	623.8	2.22	0.128
		Litters ~ log(GC) + R+ Td	623.8	2.23	0.127

Appendix E



Figur 11. Distribution and pattern of cortisol consentration at different plates at a) year 2014 -2018 b) origin of juvenile Arctic foxes.



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