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Total triiodothyronine and glucocorticoid metabolite levels in faecal samples are independent of colour morph in the Arctic fox (*Vulpes lagopus*)

Master's thesis in Biology

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Abstract

Animals living in the arctic often have physiological adaptations that allow them to cope with both extreme and variable environmental conditions throughout the year. It has been suggested that fur colour phenotype could be the basis of adaptations to the environmental conditions that the Arctic fox (*Vulpes lagopus*) experience. In two subpopulations in Norway, colour morphs of the Arctic fox seemingly influence their fitness with the blue morph having higher reproductive success and survival than the white morph. Previous studies investigating colour polymorphism in Arctic fox have revealed possible effects of melanism on metabolism and the stress response, but the exact physiological mechanisms mediating these effects remain unknown. The purpose of this study was to examine whether colour morphs influence total triiodothyronine (TT₃) and glucocorticoid concentrations in the Arctic fox. To investigate this, faecal samples were collected from free-living Arctic fox and analysed for TT₃ and faecal glucocorticoid metabolites (fGCM). The results showed that neither faecal TT₃ or fGCM concentrations were influenced by colour morph, suggesting that other physiological mechanisms related to colour dimorphism are potentially responsible for the observed fitness differences in the two colour morphs of Arctic fox.

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1 Introduction

Arctic animals are exposed to both extreme and variable environmental conditions throughout the year. In contrast with summer conditions, arctic animals experience low temperatures and harsh winter conditions, along with a reduction in food availability during the winter season. Temperatures can stay at -40°C for long prolonged periods of time (Blix, 2016). Heavy snowfall can affect winter migration and animals' ability to move long distances (Penczykowski et al., 2017), while snow cover and hibernation of potential prey animals reduce food accessibility (Bartoń & Zalewski, 2007; Warret Rodrigues & Roth, 2023). Exposure to these extreme environmental variations between seasons requires specific physiological adaptations that allow animals such as the Arctic fox (*Vulpes lagopus*) to cope with these environmental challenges (Blix, 2016; Fuglei et al., 2000). Arctic animals exhibit a range of adaptations to the arctic environment. Adaptions to the arctic include for example reduced activity in seasons with unfavourable environmental conditions, as seen in polar bears (*Ursus maritimus*) during summer (Lunn & Stirling, 1985), and hibernating arctic ground squirrel (*Spermophilus parryii kennicottii*) during winter (Buck & Barnes, 1999). Further, accumulation of fat storage to use as energy reserves and insulation, e.g. in Arctic foxes (Fuglei & Øritsland, 1999; Prestrud & Nilssen, 1992; Underwood & Reynolds, 1980) and polar bears (Atkinson et al., 1996), supports periods of reduced food availability and low ambient temperatures. Moreover, multiple arctic animals also exhibit physical adaptations, such as a round body shape and short limbs that reduces heat loss, as seen in Arctic hares (*Lepus arcticus*), Arctic foxes and reindeer (*Rangifer tarandus*) (Callaghan et al., 2004), as well as seasonal changes in fur insulation as in reindeer (Timisjärvi et al., 1984), Arctic foxes (Underwood & Reynolds, 1980) and rodents (Walsberg, 1991).

It has been suggested that fur colour phenotype could be the basis of adaptations to the environmental conditions that the Arctic fox experience (Di Bernardi et al., 2021; Hersteinsson & Macdonald, 1992; Pagh & Hersteinsson, 2008). The Arctic fox exhibits colour polymorphism and undergoes a seasonal moult where the foxes obtain either white or blue winter fur (Chesemore, 1970). Which colour morph the individuals acquire is genetically determined (Våge et al., 2005). Studies investigating several fitness parameters, such as survival, litter size and breeding propensity, revealed that in two subpopulations in Norway, the blue colour morph had higher fitness compared to the white morph (Di Bernardi et al., 2021; Tietgen et al., 2021). The authors suggested that the higher fitness of the blue morph might be due to physiological traits linked to fur colour (Di Bernardi et al., 2021). However, they did not elaborate and specify

the exact physiological mechanisms underlying the fitness differences observed between the colour morphs.

Melanin-based variation in behavioural and physiological traits has been observed especially in vertebrates (Ducrest et al., 2008), e.g. darker pigmented barn owls (*Tyto alba*) having stronger immune response (Roulin et al., 2000) and greater parasite resistance (Roulin et al., 2001), higher degree of antipredator behaviour in darker Spanish terrapins (*Mauremys leprosa*) (Ibáñez et al., 2016), and African lions (*Panthera leo*) with darker manes being more aggressive and sexually active (West & Packer, 2002). A possible explanation for melanism based variation in physiology and behaviour lies in the melanocortin system (Ducrest et al., 2008). The melanocortin system consists of the posttranslational products of the proopiomelanocortin (POMC) gene, called melanocortins, and the responses these products initiate (Ducrest et al., 2008). Melanocortins can act on five different melanocortin receptors, named Melanocortin 1-5 Receptors (MC1-5R), and each receptor is responsible for several behavioural or physiological functions. Binding of melanocortins to the Melanocortin 1 Receptor (MC1R) gene determines colour morph in the Arctic fox (Tietgen et al., 2021). Since melanocortins also bind to MC2-5R, this can result in covariation between colouration and other phenotypic traits, possibly also in the Arctic fox (Ducrest et al., 2008; Tietgen et al., 2021). Initiation of the stress response and steroid production is triggered by the binding of melanocortins to the MC2R (Ducrest et al., 2008). Additionally, binding to the MC3R and MC4R receptors is associated with increased energy expenditure, reduced food intake, regulation of glucose homeostasis, and, in the case of the MC4R, increased stress resistance. Furthermore, binding to the MC5R receptor has been linked to heightened aggressiveness and exocrine gland activity. A comprehensive review of colour dimorphic species revealed that several factors related to the stress response and energy homeostasis were impacted by melanism, including reduced food intake and adipose tissue volume in darker morphs, as well as elevated thyroid hormone activities, metabolic rate and diet-induced thermoregulation (Ducrest et al., 2008). Thus, Ducrest et al. (2008) suggests that animals with colour polymorphism are expected to have different activities of the traits induced by the melanocortin receptors. Further, the exact physiological mechanisms that could potentially confer a fitness advantage to the blue colour morph of the Arctic fox have yet to be investigated. Given the link between fur colour and melanism, as well as the proposed associations between the melanocortin system and other physiological traits (Ducrest et al., 2008; Roulin & Ducrest, 2011), it seems intuitive to investigate differences in these physiological traits between the white and the blue colour morphs in the Arctic fox.

To be able to achieve a high and stable body temperature, endotherms have high metabolic rates, resulting in a high demand for energy to maintain the required metabolic activity (Bennett & Ruben, 1979). During winter, the Arctic fox is exposed to both low temperatures and reduced food availability (Fuglei et al., 2000), resulting in high thermal conductance and less energy available to maintain a high metabolic rate. To cope with this, the Arctic fox has been shown to exhibit seasonal metabolic depression, allowing them to consume and expend less energy to maintain crucial physiological processes (Fuglei & Øritsland, 1999; Fuglei et al., 2000). At the same time, the Arctic fox could possibly also increase their metabolic activity at low ambient temperature to maintain their core body temperature (Fuglei & Øritsland, 1999). A genetic analysis exploring the basis of melanism in the Arctic fox revealed a possible genetic link between fur colouration, metabolic control and energy expenditure (Tietgen et al., 2021). Yet, it remains unclear if the two colour morphs exhibit different metabolic activities during periods with low temperatures and reduced food availability. Thyroid hormones influence the metabolism of endothermic animals, and therefore are often used as proxies for animals' metabolic state (Behringer et al., 2018; Hulbert, 2000; Silva, 2006). Circulating thyroid hormone levels is regulated by the hypothalamus-pituitary-thyroid (HPT)-axis through negative feedback mechanisms (Zoeller et al., 2007). Thyrotropin-releasing hormone (TRH) is released from the hypothalamus, signalling thyroid-stimulating hormone (TSH) release from the pituitary gland, resulting in production and release of thyroxine (T_4) and the biologically active triiodothyronine (T_3) from the thyroid gland. Thyroid hormones are important for mediating facultative thermoregulation in endothermic animals, e.g. by regulating basal metabolic rate (BMR) and heat production in brown adipose tissue (BAT) (Hulbert, 2000). During periods of starvation, the production of thyroid hormones is reduced, leading to metabolic depression (Flier et al., 2000). Hence, investigating potential differential thyroid hormone levels comparing the two colour morphs can reveal a potential association between colour dimorphism and energy homeostasis in the Arctic fox.

In addition to having effects on the energetics of an animal, Ducrest et al. (2008) also argue that melanism influences the animals' stress response, for instance by affecting circulating glucocorticoid levels. Glucocorticoids are hormones that are involved in energy mobilization and the stress response (Charmandari et al., 2005; Magomedova & Cummins, 2015). Circulating glucocorticoid levels are regulated by the hypothalamus-pituitary-adrenal glands (HPA)-axis through negative feedback mechanisms (Charmandari et al., 2005). Corticotropin-releasing hormone (CRH) released from the hypothalamus signals release of adrenocorticotrophic hormone (ACTH) from the pituitary, which act on the adrenal glands and

stimulate the production and release of glucocorticoids. It has been suggested that pigmentation can influence stress response, due to observations of darker pigmented individuals having both lower baseline levels of glucocorticoids and a shorter recovery period before returning to baseline levels of glucocorticoids after being exposed to stress (Roulin & Ducrest, 2011). Additionally, as a metabolic hormone, glucocorticoids influence glucose homeostasis by inducing energy mobilization (Charmandari et al., 2005; Magomedova & Cummins, 2015). Increased glucocorticoid levels can be observed for example during periods of starvation or during periods of increased energetic demand, which both are situations when there is a larger requirement for utilizing energy reserves (Wingfield & Romero, 2010). Thus, comparing baseline glucocorticoid levels of the two Arctic fox colour morphs might elucidate potential differences in their energy homeostasis and their tolerance to environmental stress.

The aim of this study is to investigate the physiological mechanisms underlying the observed fitness difference between the two Arctic fox colour morphs. Specifically, we will investigate variations in faecal thyroid hormone and glucocorticoids as proxies for metabolic activities and energy utilization between the white and blue colour morphs of the Arctic fox. We predict that (1) the blue colour morph will have higher levels of total T_3 (TT_3) and thus potentially better able to regulate metabolism for facultative thermoregulation and energy balance, and (2) the blue colour morph will have higher glucocorticoid levels and thus potentially better able to mobilize energy reserves.

2 Methodology

2.1 Sample selection

Faecal samples were collected from free living Arctic foxes in Norway in the period of January - May in both 2019 ($n_{2019}=140$) and 2020 ($n_{2020} = 287$), originating from a total of 140 individuals. The samples were collected from both blue ($n_{blue} = 120$) and white ($n_{white} = 307$) colour morphs, and multiple of the individuals were sampled repeatedly ($n_{faecal} = 427$). Samples were collected from nine subpopulations in Norway: Hardangervidda ($n_{Hardangervidda} = 94$), Snøhetta ($n_{Snøhetta} = 117$), Hestkjølen ($n_{Hestkjølen} = 31$), Blåfjellet ($n_{Blåfjellet} = 39$), Kjølifjellet/Sylane ($n_{Kjølifjellet/Sylane} = 32$), Børgefjell ($n_{Børgefjell} = 45$), and Varangerhalvøya ($n_{Varangerhalvøya} = 38$). Of each faecal sample, 0.5 g was extracted with 5 mL 80% methanol before they were analysed for faecal glucocorticoid metabolite (fGCM) concentration using enzyme immunoassay (EIA) at the University of Veterinary Medicine in Vienna (Larm et al., 2021). The TT_3 assay was conducted at the Norwegian University of Science and Technology. While all the collected samples were analysed for fGCM concentration, a subset of samples were

selected for a TT₃ assay to compare the concentration of fGCM and TT₃ in the faeces of blue and white foxes. Due to the smaller sample size of blue foxes, all blue individuals were included and equally as many white individuals. Several criteria were considered when selecting samples for the TT₃ assay: about equal sex ratio, equal white:blue ratio, samples from most of the subpopulations, and a short range of sample period to avoid seasonal effects. One sample was selected per individual for the TT₃ assay. The sample size for the TT₃ assay was 70 samples, with 1:1 ratio of white and blue colour morphs, including 30 female samples ($n_{\text{female}} = 30$: $n_{\text{blue}} = 13$, $n_{\text{white}} = 17$ white), and 40 male samples ($n_{\text{male}} = 40$: $n_{\text{blue}} = 22$, $n_{\text{white}} = 18$). A summary of the sample sizes can be found in Table S1 in the Supplementary material.

2.2 TT₃ assay using enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) was used to quantify TT₃ concentrations in faecal sample extracts, using DetectX Triiodothyronine (T₃) Enzyme Immunoassay kits (Ann Arbor, Michigan). The TT₃ assay was performed according to the protocol provided with the ELISA kits. The faecal sample extracts were diluted with a dilution factor of 20 with Assay buffer so that the methanol concentration in the dilutions were < 5%. Dilutions were loaded into 96-well microtiter plates coated with donkey anti-sheep IgG. The optical density of each microtiter plate was read at 450 nm wavelength using a Biotek Cytation 5 cell Imaging Multimode Reader. After reading optical density of all the samples, samples that had TT₃ concentration below detection limit were rerun using double the volume of the diluted samples in the wells. The interassay and intraassay CV's were 11.63% and 5.97%, respectively.

2.3 Statistical analysis

All statistical analyses were performed using R version 4.2.2, RStudio version 2023.03.0+386 (R Core Teams, 2023). A summary of the models and the statistical output can be found in Table S2 in the Supplementary material. Normality of the residuals was inspected using Q-Q plots and by checking fitted values against residuals. Concentrations of TT₃ and fGCM were log₁₀-transformed to achieve normally distributed data before statistical analysis. Interaction between colour and sex was tested for each model, and the interaction term was not included in the model if there were no significant interactions.

For the TT₃ data, a generalized linear model was conducted, with log₁₀-transformed TT₃ values as the response variable, colour and sex as main effects, and controlling for sample year

and subpopulation. Sample date was not included as a covariate due to the short range of sampling period for the selected sample subset for the TT₃ assay.

For the fGCM data, a linear mixed effect model (LME4-package in R) was conducted to control for repeated measurements, with log₁₀-transformed fGCM values as the response variable, colour and sex as main effects, controlling for sample year, subpopulation and sample date, and with individual as a random factor.

3 Results

3.1 Effects of colour, sex, sample year, and subpopulation on TT₃ levels

There was no significant interaction between colour and sex on TT₃ levels ($F_{1,59}=1.64$, $p>0.05$). There was also no effect of colour morph ($F_{1,68}=0.11$, $p>0.05$; Figure 1), sex ($F_{1,67}=0.0059$, $p>0.05$; Figure 1), or sample year ($F_{1,66}=0.63$, $p>0.05$) on TT₃ levels in the collected faecal samples of the Arctic fox. The analysis demonstrated a significant effect of subpopulation on TT₃ levels ($F_{6,60}=2.31$, $p=0.0451$). However, post-hoc tukey's pairwise comparison revealed no significant differences between the subpopulations examined. The adjusted R² of the model was 0.075.

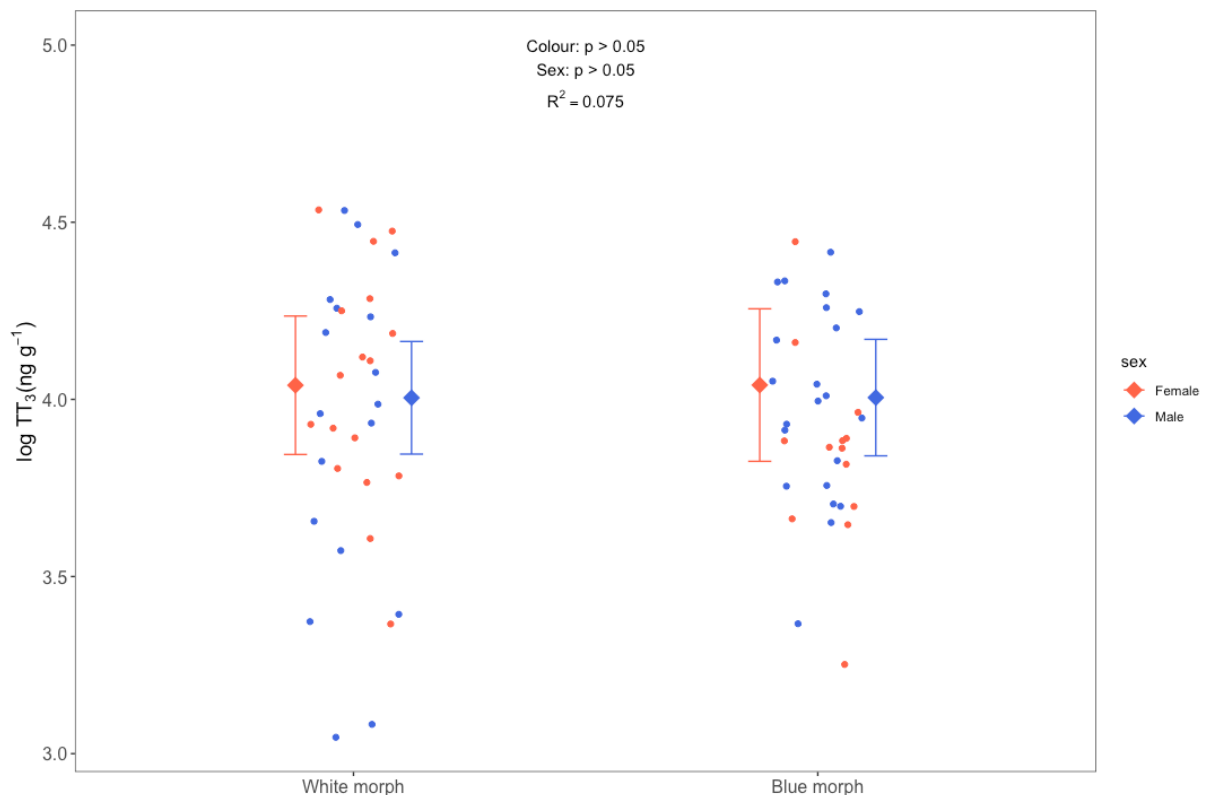


Figure 1: The effect of colour and sex on log₁₀-transformed TT₃ levels, in which both were non-significant. Red circles show female TT₃ values, and blue circles show male TT₃ values. The diamond-shaped points are the least square means, with corresponding standard error of the means bars.

3.2 Effects of colour, sex, time, sample year, and subpopulation on fGCM levels

There was no significant interaction between colour and sex on fGCM levels ($F_{1,136}=0.56$, $p>0.05$). Furthermore, there was no effect of colour ($F_{1,137}=0.19$, $p>0.05$; Figure 2) or sex ($F_{1,137}=0.17$, $p>0.05$; Figure 2) on Arctic fox fGCM levels in the collected faecal samples. However, there was a significant effect of sampling date ($F_{1,278}=17.25$, $p<0.0001$; Figure 3) on fGCM levels, where concentration decreased over time for the sample period of January - May. Moreover, there was a significant effect of sample year on fGCM concentration ($F_{8,278}=4.61$, $p=0.0326$; Figure 3), with faecal samples collected in 2020 having higher fGCM levels compared to samples collected in 2019. Lastly, there was a significant effect of subpopulation on fGCM levels ($F_{1,278}=2.045$, $p=0.0498$). However, post-hoc tukey's pairwise comparison showed no significant differences between the subpopulations. The adjusted R^2 of the model was 0.43.

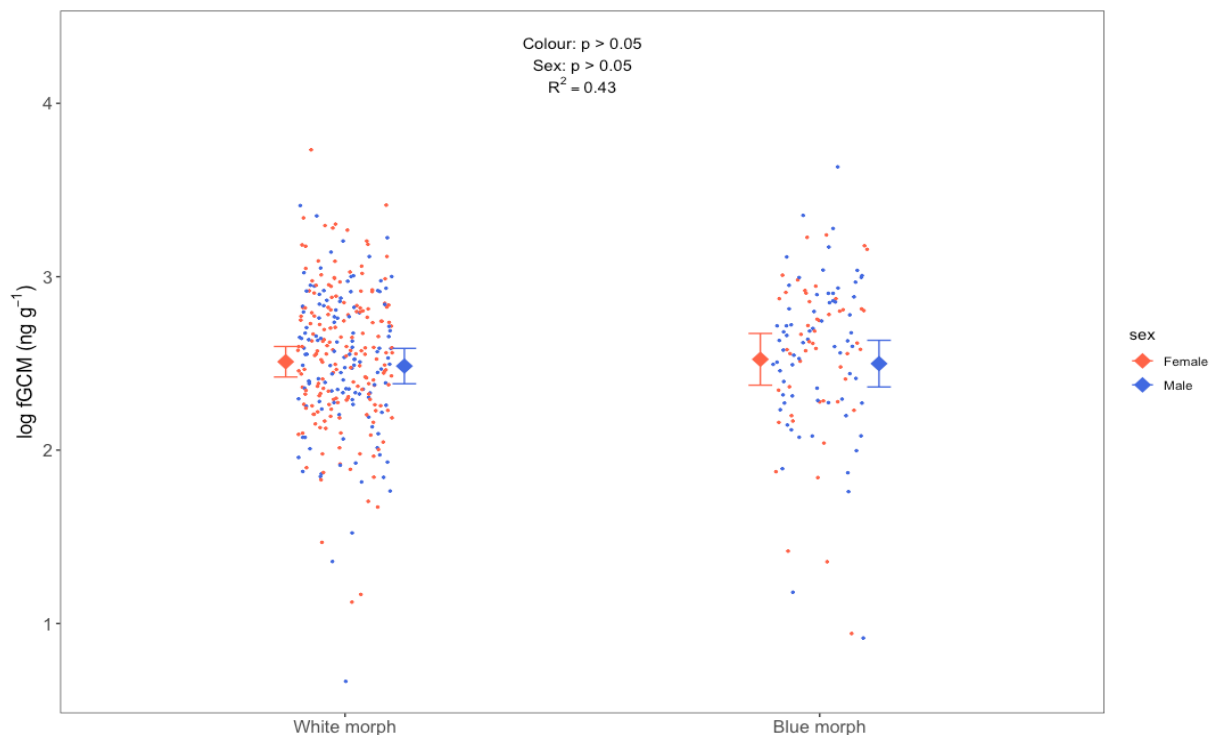


Figure 2: The effect of colour and sex on \log_{10} -transformed fGCM levels, in which both were non-significant. Red circles show female fGCM values, and blue circles show male fGCM values. Diamond-shaped points are the least square means, with corresponding standard error of the means bars.

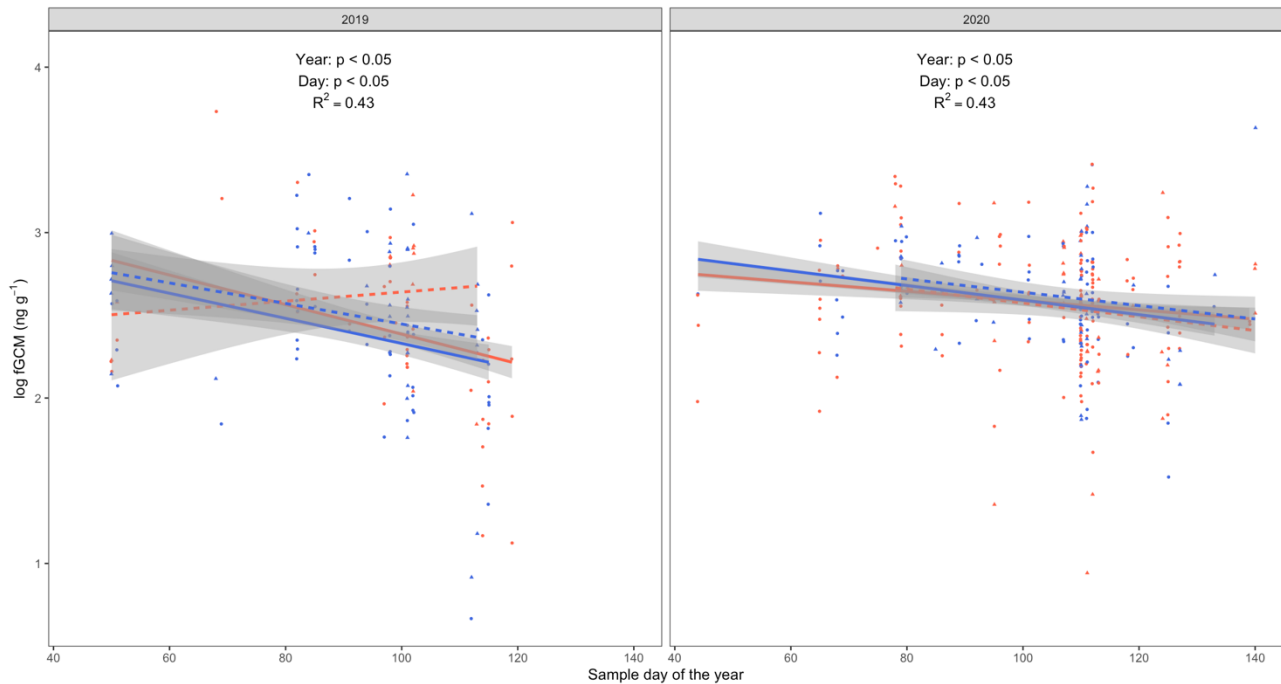


Figure 3: The effect of sample day and year on \log_{10} -transformed fGCM concentrations, in which both were significant. Red data points show female measurements, and blue data points show male measurements. Circles show data points for white morphs and triangles for blue morphs. The fitted lines are based on a mixed effect model. Stapled lines show the fitted line for the blue morph, and the solid line show the fitted line for the white morph. 95% confidence intervals are shown in grey.

4 Discussion

This study investigated whether TT_3 and glucocorticoid production in the Arctic fox is dependent on colour morph. This was done by comparing TT_3 levels and fGCM levels in faecal samples collected from free living Arctic foxes in Norway. The main findings suggest that TT_3 and fGCM concentrations are independent of fur colour in the Arctic fox, as there was no significant difference in TT_3 and fGCM levels comparing blue and white foxes. There was, however, an effect of sampling date on fGCM concentration in both colour morphs, indicating that Arctic foxes reduced their glucocorticoid production throughout the period of January - May.

In contrast to our initial hypothesis, we found no difference in TT_3 levels between the two Arctic fox colour morphs. Specifically, the lack of a correlation between Arctic fox colour morph and TT_3 levels suggests that there was no difference between the two colour morphs in thyroid hormone induced metabolic activity, such as facultative thermoregulation. This finding potentially rules out the association between thermoregulatory differences in the two colour morphs and their differential survival rate in nature as reported by Di Bernardi et al. (2021). It

is known that darker surfaces absorb more heat from the environment and radiation, and thereby pigmentation is important for passive heat gain especially in many ectotherms (Trullas et al., 2007). Conversely, studies on arctic mammals have found minimal importance of winter pelage colouration for heat absorption due to the effect of fur insulation (Dawson et al., 2014; Walsberg, 1991), and with the Arctic fox being mainly nocturnal (Audet et al., 2002; Thierry et al., 2020), heat gain by solar radiation presumably has a negligible thermal effect.

Multiple studies have found similar evidence of colour polymorphism being independent of metabolic activity, e.g. in the Common Wall lizard (*Podarcis muralis*) (Gomes et al., 2022), guppies (*Poecilia reticulata*) (Santostefano et al., 2019) and the Gouliidan finch (*Erythrura gouldiae*) (Buttemer et al., 2021). Contrarily, there is also evidence for individuals with a higher degree of pigmentation having higher metabolic rate, as in great tits (*Parus major*) with wider breast-stripes (Røskaft et al., 1986), and pied flycatchers (*Ficedula hypoleuca*) with darker plumage (Røskaft et al., 1986). Since metabolism was not directly measured in this experiment, there could perhaps still be melanism based differences in metabolic activity not explained by faecal T₃.

Contrary to our initial hypotheses, there was no difference in fGCM levels between the two colour morphs. Specifically, the lack of a correlation between colour morph and glucocorticoid levels suggests that the two colour morphs have similar degrees of glucocorticoid mediated utilization of energy reserves, and are equally susceptible to environmental stress. This finding potentially rules out that there is an association between energy mobilization in the two colour morphs and their differential success in nature. Some studies have reported more pigmented individuals having lower baseline levels of glucocorticoids compared to less pigmented individuals, like in barn owls (Roulin & Ducrest, 2011) and white throated sparrows (*Zonotrichia albicollis*) (Horton & Holberton, 2010). Further, Almasi et al. (2010) found that more pigmented barn owls return to baseline glucocorticoid levels faster after stress exposure compared to less pigmented individuals. On the other hand, several studies have found that melanism is not necessarily correlated with glucocorticoid levels or the stress response, as in Eastern Box Turtles (*Terrapene carolina carolina*) (Carlson & Robinson, 2022), guppies (Santostefano et al., 2019), and barn swallows (*Hirundo rustica erythrogaster*) (Jenkins et al., 2013). It seems therefore that the effect of melanism on glucocorticoid levels is species dependent, and possibly not present in the Arctic fox.

Although the higher fitness of the blue foxes cannot be explained by differences in thyroid hormones and glucocorticoids, there might be other aspects of the melanocortin system that has

fitness-related effects in the Arctic fox. For example, hormone sensitivity can potentially be melanism dependent. In house sparrows (*Passer domesticus*), a negative correlation between area of the pigmented chest bib and density of glucocorticoid receptors present in the chest bib skin suggests more pigmented individuals were less responsive to a rise in glucocorticoid levels (Lattin & Romero, 2013). Moreover, glucocorticoid administration in barn owls resulted in a greater reduction in parental investment in less pigmented individuals, a common characteristic of experienced stress in birds (Almasi et al., 2008). Another possible effect of colour morph is the ability to cope with poor environmental conditions. As observed in Tietgen et al. (2021), blue morphs produced more recruits and had higher survival compared to white morphs in periods of low food availability, suggesting that blue foxes might cope better in poor environmental conditions compared to white foxes. A similar observation was made by Dreiss et al. (2010), who reported that melanism is positively correlated with the ability to cope with starvation due to effects on energy homeostasis in barn owls. Further, Di Bernardi et al. (2021) observed that the blue foxes have higher breeding probability, although this seems to mainly be the case for female individuals (Tietgen et al., 2021). Hence, there is a possibility that there are other aspects of the melanocortin system that affects female breeding probability and success. Roulin et al. (2003) observed this in tawny owls, where grey females produce higher quality offspring while reddish females breed more often. Melanism has already been reported to confer greater sperm quality in blue male Arctic foxes (Stasiak et al., 2019), further supporting that reproductive success potentially is influenced by colour morph.

The observed effect of sample year on fGCM levels, with higher levels in 2020 compared to 2019, suggest that glucocorticoid production in the Arctic fox is affected by temporal fluctuations in environmental factors. There might be several explanations for the observed effect of sample year on glucocorticoid levels, such as variation in food availability, reproduction, and temperature. The Arctic fox mainly feeds on rodents, specifically lemmings (*Lemmus lemmus*) (Angerbjorn et al., 1999; Elmhagen et al., 2002), a species that follow a quite distinct 3-5 year population cycles (Chr, 1999). This would indicate that foxes have elevated glucocorticoid levels in years with low lemming density due to greater utilization of energy reserves, and thereby possibly are more susceptible to stress in these periods (Magomedova & Cummins, 2015). However, the observed effect of sample year is not in line with this expectation, as the rodent population in the examined subpopulations was generally lower in 2019 compared to 2020 (unpublished data, *Norwegian Institute for Nature Research*). However, it should be noted that rodent phases can be region and subpopulation specific and thus warrant further investigations. This suggests that food availability alone does not explain

glucocorticoid levels in the Arctic fox. Moreover, breeding occurrence was reported for some of the individuals sampled in 2020, but not for any of the individuals sampled in 2019 (unpublished data, *Norwegian Institute for Nature Research*). Potential temperature differences in the two respective years could also have affected the glucocorticoid levels, as a lower temperature usually is correlated with an increase in glucocorticoid production (de Bruijn & Romero, 2018). Most likely, the observed effect of year is explained by a combination of several factors, such as food availability, reproduction and temperature fluctuations.

fGCM concentration decreased throughout the sampling period from January-May in both sample years, independent of colour morph. Reduced glucocorticoid production within the sampling period could be related to increasing food availability going from winter to spring. Specifically, higher glucocorticoid levels in winter reflects the need to use stored energy reserves as an energy source due to low food availability. Even though the sampling period did not include samples from summer and autumn, the decrease in fGCM levels with increasing daylight length suggests that Arctic foxes have a circannual pattern of glucocorticoid production, possibly affected by food availability. Elevated glucocorticoid levels in winter with lower levels in summer has also been reported in other fox species, e.g. island foxes (*Urocyon littoralis*) (Kozłowski et al., 2020). Diminishing glucocorticoid production from January - May also coincides with the Arctic fox reducing its fat reserves going from winter to summer (Fuglestad et al., 2006; Prestrud & Nilssen, 1992), suggesting that the foxes experience an ever decreasing risk of sudden starvation in this period (Blix, 2016; Prestrud & Nilssen, 1992). This is supported by Fuglei et al. (2000), who reported a starvation induced increase in circulating free fatty acid concentrations in the Arctic fox, indicating a greater requirement for energy mobilization of fat reserves during periods of starvation. Similar patterns of seasonal changes in body mass has been observed in other arctic species, like reindeer (Blix, 2016) and Spizbergen ptarmigan (*Lagopus mutus hyperboreus*) (Mortensen et al., 1983), with body fat reserves dissipating when there is an increasing chance of finding food after a period of low food availability in the winter.

To our knowledge, this is the first study that has investigated relationships between colour morph and levels of both TT_3 and glucocorticoids in the endangered Arctic fox. Along with Stasiak et al. (2019), who investigated differential sperm quality in the two colour morphs, this is one of few studies that has examined potential associations between colour morph and physiological traits in the Arctic fox. While the findings in our study suggests that there is no effect of colour morph on fGCM and TT_3 levels, potential relationships between colour morph and other physiological traits in the Arctic fox warrant further investigation.

Faecal sampling is a helpful tool for conducting non-invasive sample collection for hormone assays, making it possible to collect samples also of endangered species like the Arctic fox with minimal, if any, effects on the animals sampled (Behringer et al., 2018; Larm et al., 2021; McDonald et al., 2018). Due to the protected status of the Arctic fox, there are limited alternative sampling methods that allow data collection of a similar quantity compared to collecting faecal samples, as well as provide a cumulative hormone measurement to investigate inherent contrasts of hormone levels.

One limitation of this study is the measurement of TT_3 concentration instead of free versus bound T_3 concentrations (Behringer et al., 2018). Circulating T_3 , being a hydrophobic molecule, is mainly transported bound to carrier proteins (Hulbert, 2000). However, it is only free T_3 that is biologically active to exert its physiological functions on target tissues, and thus the most relevant measurement when studying metabolic activity (Behringer et al., 2018; Tomasi, 1991). Hence, since free T_3 was not measured in this study, there is a possibility that TT_3 concentrations comparing individuals are similar, but with different ratio of free and bound T_3 (Hulbert, 2000; Keestra et al., 2021; McNABB et al., 1984). Measuring free T_3 instead of, or together with total T_3 would perhaps give a better view of potential differences in metabolic activity in the two Arctic fox colour morphs. However, this could only be achieved using blood samples and not faecal samples (Malisch & Breuner, 2010).

Another limitation of this study is the method for faecal sample collection. The faecal samples were collected sporadically, which caused a pooling of samples on certain dates within the sampling period. Thus, samples collected in the field on the same day and in the same area have most likely been exposed to unknown and distinctive environmental conditions, like different temperature, precipitation and solar radiation (Millsbaugh & Washburn, 2004). Comparing faecal samples collected on the same day is therefore not necessarily controlling for defecation time, and both TT_3 and fGCM levels might have been drastically altered (Behringer et al., 2018; Millsbaugh & Washburn, 2004). Furthermore, the fact that within individual variation is unknown is a confounding factor when using faecal samples, since this might have unknown effect on the hormone content in the faecal samples (Palme, 2019). Interpretation of hormone concentrations when comparing faecal samples will therefore be affected.

Future studies should further examine differences in metabolic activity between the two colour morphs by measuring metabolism more directly through for example flow-through respirometry, and measure free T_3 levels in the Arctic fox. Preferably, faecal samples should be collected shortly after defecation to ensure minimal environmental effects on hormone levels in the samples, and within 48 hours after defecation to avoid hormonal degradation, at least for

fGCM measurements (Larm et al., 2021). Faecal samples should also be collected in a more continuous manner over the course of the sampling period to increase the certainty of the effect of sampling date on hormone levels, and preferably covering more of the annual cycle. Possible effects of melanism on sex hormones in females would also be of interest due to the tendency of blue females to breed more often and having larger litters, especially in years with poor environmental conditions.

5 Conclusion

The study found no significant difference in faecal TT_3 or fGCM concentrations between the two colour morphs of the Arctic fox, indicating that the greater success of the blue morph is unlikely to be due to melanism-based effects thyroid hormone and glucocorticoids. Thus, the results of the study were not in line with the hypotheses. However, there may still be other melanism-related effects that contribute to differences in fitness between the two morphs, such as variations in sensitivity to glucocorticoids and thyroid hormones, differences in reproductive strategies and success, and effects on traits that impact the foxes' ability to cope with certain environmental conditions.

References

- Almasi, B., Jenni, L., Jenni-Eiermann, S., & Roulin, A. (2010). Regulation of stress response is heritable and functionally linked to melanin-based coloration. *Journal of Evolutionary Biology*, *23*(5), 987-996.
- Almasi, B., Roulin, A., Jenni-Eiermann, S., & Jenni, L. (2008). Parental investment and its sensitivity to corticosterone is linked to melanin-based coloration in barn owls. *Hormones and behavior*, *54*(1), 217-223.
- Angerbjörn, A., Tannerfeldt, M., & Erlinge, S. (1999). Predator-prey relationships: arctic foxes and lemmings. *Journal of Animal Ecology*, *68*(1), 34-49.
- Atkinson, S. N., Nelson, R. A., & Ramsay, M. A. (1996). Changes in the body composition of fasting polar bears (*Ursus maritimus*): the effect of relative fatness on protein conservation. *Physiological Zoology*, *69*(2), 304-316.
- Audet, A. M., Robbins, C. B., & Larivière, S. (2002). Alopex lagopus. *Mammalian species*, *2002*(713), 1-10.
- Bartoń, K. A., & Zalewski, A. (2007). Winter severity limits red fox populations in Eurasia. *Global Ecology and Biogeography*, *16*(3), 281-289.
- Behringer, V., Deimel, C., Hohmann, G., Negrey, J., Schaebs, F. S., & Deschner, T. (2018). Applications for non-invasive thyroid hormone measurements in mammalian ecology, growth, and maintenance. *Hormones and behavior*, *105*, 66-85.
- Bennett, A. F., & Ruben, J. A. (1979). Endothermy and activity in vertebrates. *Science*, *206*(4419), 649-654.
- Blix, A. S. (2016). Adaptations to polar life in mammals and birds. *Journal of Experimental Biology*, *219*(8), 1093-1105.
- Buck, C. L., & Barnes, B. M. (1999). Annual cycle of body composition and hibernation in free-living arctic ground squirrels. *Journal of Mammalogy*, *80*(2), 430-442.
- Buttemer, W. A., Careau, V., Chappell, M. A., & Griffith, S. C. (2021). Metabolic rates of aggressive and submissive phenotypes are colour blind in the polymorphic Gouldian finch. *Journal of Experimental Biology*, *224*(15), jeb242577.
- Callaghan, T. V., Björn, L. O., Chernov, Y., Chapin, T., Christensen, T. R., Huntley, B., Ims, R. A., Johansson, M., Jolly, D., & Jonasson, S. (2004). Biodiversity, distributions and adaptations of Arctic species in the context of environmental change. *AMBIO: A Journal of the Human Environment*, *33*(7), 404-417.

- Carlson, B. E., & Robinson, W. L. (2022). Trait Covariances in Eastern Box Turtles Do Not Support Pleiotropic Effects of the Melanocortin System on Color, Behavior, and Stress Physiology. *Journal of Herpetology*, *56*(4), 478-488.
- Charmandari, E., Tsigos, C., & Chrousos, G. (2005). Endocrinology of the stress response. *Annu. Rev. Physiol.*, *67*, 259-284.
- Chesemore, D. L. (1970). Notes on the pelage and priming sequence of arctic foxes in northern Alaska. *Journal of Mammalogy*, *51*(1), 156-159.
- Chr, N. (1999). Population cycles in voles and lemmings: density dependence and phase dependence in a stochastic world. *Oikos*, 427-461.
- Dawson, T. J., Webster, K. N., & Maloney, S. K. (2014). The fur of mammals in exposed environments; do crypsis and thermal needs necessarily conflict? The polar bear and marsupial koala compared. *Journal of Comparative Physiology B*, *184*, 273-284.
- de Bruijn, R., & Romero, L. M. (2018). The role of glucocorticoids in the vertebrate response to weather. *General and comparative endocrinology*, *269*, 11-32.
- Di Bernardi, C., Thierry, A. M., Eide, N. E., Bowler, D. E., Rød-Eriksen, L., Blumentrath, S., Tietgen, L., Sandercock, B. K., Flagstad, Ø., & Landa, A. (2021). Fitness and fur colouration: Testing the camouflage and thermoregulation hypotheses in an Arctic mammal. *Journal of Animal Ecology*, *90*(5), 1328-1340.
- Dreiss, A., Henry, I., Ruppli, C., Almasi, B., & Roulin, A. (2010). Darker eumelanic barn owls better withstand food depletion through resistance to food deprivation and lower appetite. *Oecologia*, *164*, 65-71.
- Ducrest, A.-L., Keller, L., & Roulin, A. (2008). Pleiotropy in the melanocortin system, coloration and behavioural syndromes. *Trends in ecology & evolution*, *23*(9), 502-510.
- Elmhagen, B., Tannerfeldt, M., & Angerbjörn, A. (2002). Food-niche overlap between arctic and red foxes. *Canadian Journal of Zoology*, *80*(7), 1274-1285.
- Flier, J. S., Harris, M., & Hollenberg, A. N. (2000). Leptin, nutrition, and the thyroid: the why, the wherefore, and the wiring. *The Journal of clinical investigation*, *105*(7), 859-861.
- Fuglei, E., & Øritsland, N. (1999). Seasonal trends in body mass, food intake and resting metabolic rate, and induction of metabolic depression in arctic foxes (*Alopex lagopus*) at Svalbard. *Journal of Comparative Physiology B*, *169*, 361-369.
- Fuglei, E., Aanestad, M., & Berg, J. (2000). Hormones and metabolites of arctic foxes (*Alopex lagopus*) in response to season, starvation and re-feeding. *Comparative*

- Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 126(2), 287-294.
- Fuglestad, B. N., Haga, Ø. E., Folkow, L. P., Fuglei, E., & Blix, A. S. (2006). Seasonal variations in basal metabolic rate, lower critical temperature and responses to temporary starvation in the arctic fox (*Alopex lagopus*) from Svalbard. *Polar Biology*, 29, 308-319.
- Gomes, V., Žagar, A., Lanuza, G. P. i. d., Simčič, T., & Carretero, M. A. (2022). Is it function or fashion? An integrative analysis of morphology, performance, and metabolism in a colour polymorphic lizard. *Diversity*, 14(2), 116.
- Hersteinsson, P., & Macdonald, D. W. (1992). Interspecific competition and the geographical distribution of red and arctic foxes *Vulpes vulpes* and *Alopex lagopus*. *Oikos*, 505-515.
- Horton, B. M., & Holberton, R. L. (2010). Morph-specific variation in baseline corticosterone and the adrenocortical response in breeding white-throated sparrows (*Zonotrichia albicollis*). *The Auk*, 127(3), 540-548.
- Hulbert, A. (2000). Thyroid hormones and their effects: a new perspective. *Biological Reviews*, 75(4), 519-631.
- Ibáñez, A., Pellitteri-Rosa, D., Sacchi, R., López, P., & Martín, J. (2016). Melanin-based coloration covaries with hiding and exploratory behavior in male Spanish terrapins. *Ethology*, 122(1), 30-36.
- Jenkins, B. R., Vitousek, M. N., & Safran, R. J. (2013). Signaling stress? An analysis of phaeomelanin-based plumage color and individual corticosterone levels at two temporal scales in North American barn swallows, *Hirundo rustica erythrogastrer*. *Hormones and behavior*, 64(4), 665-672.
- Keestra, S., Höggqvist Tabor, V., & Alvergne, A. (2021). Reinterpreting patterns of variation in human thyroid function: An evolutionary ecology perspective. *Evolution, Medicine, and Public Health*, 9(1), 93-112.
- Kozłowski, C. P., Clawitter, H., Guglielmino, A., Schamel, J., Baker, S., Franklin, A. D., Powell, D., Coonan, T. J., & Asa, C. S. (2020). Factors affecting glucocorticoid and thyroid hormone production of island foxes. *The Journal of Wildlife Management*, 84(3), 505-514.
- Larm, M., Hovland, A. L., Palme, R., Thierry, A.-M., Miller, A. L., Landa, A., Angerbjörn, A., & Eide, N. E. (2021). Fecal glucocorticoid metabolites as an indicator of

- adrenocortical activity in Arctic foxes (*Vulpes lagopus*) and recommendations for future studies. *Polar Biology*, 44, 1925-1937.
- Lattin, C. R., & Romero, L. M. (2013). The size of a melanin-based plumage ornament correlates with glucocorticoid receptor concentrations in the skin of that ornament. *Biology letters*, 9(5), 20130440.
- Lunn, N., & Stirling, I. (1985). The significance of supplemental food to polar bears during the ice-free period of Hudson Bay. *Canadian Journal of Zoology*, 63(10), 2291-2297.
- Magomedova, L., & Cummins, C. L. (2015). Glucocorticoids and Metabolic Control. In *Handbook of Experimental Pharmacology* (pp. 73-93). Springer, Cham.
- Malisch, J. L., & Breuner, C. W. (2010). Steroid-binding proteins and free steroids in birds. *Molecular and cellular endocrinology*, 316(1), 42-52.
- McDonald, R. S., Roth, J. D., & Anderson, W. G. (2018). Prey cortisol affects the usefulness of fecal glucocorticoid metabolite concentration as an indicator of stress in a carnivore. *Canadian Journal of Zoology*, 96(4), 367-371.
- McNABB, F. A., LYONS, L. J., & HUGHES, T. E. (1984). Free thyroid hormones in altricial (ring doves) versus precocial (Japanese quail) development. *Endocrinology*, 115(6), 2133-2136.
- Millspaugh, J. J., & Washburn, B. E. (2004). Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *General and comparative endocrinology*, 138(3), 189-199.
- Mortensen, A., Unander, S., Kolstad, M., & Blix, A. (1983). Seasonal changes in body composition and crop content of Spitzbergen ptarmigan *Lagopus mutus hyperboreus*. *Ornis Scandinavica*, 144-148.
- Pagh, S., & Hersteinsson, P. (2008). Difference in diet and age structure of blue and white Arctic foxes (*Vulpes lagopus*) in the Disko Bay area, West Greenland. *Polar Research*, 27(1), 44-51.
- Palme, R. (2019). Non-invasive measurement of glucocorticoids: Advances and problems. *Physiology & behavior*, 199, 229-243.
- Penczykowski, R. M., Connolly, B. M., & Barton, B. T. (2017). Winter is changing: trophic interactions under altered snow regimes. *Food Webs*, 13, 80-91.
- Prestrud, P., & Nilssen, K. (1992). Fat deposition and seasonal variation in body composition of arctic foxes in Svalbard. *The Journal of Wildlife Management*, 221-233.

- Roulin, A., & Ducrest, A.-L. (2011). Association between melanism, physiology and behaviour: a role for the melanocortin system. *European journal of pharmacology*, 660(1), 226-233.
- Roulin, A., Ducret, B., Ravussin, P. A., & Altwegg, R. (2003). Female colour polymorphism covaries with reproductive strategies in the tawny owl *Strix aluco*. *Journal of Avian Biology*, 34(4), 393-401.
- Roulin, A., Jungi, T. W., Pfister, H., & Dijkstra, C. (2000). Female barn owls (*Tyto alba*) advertise good genes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 267(1446), 937-941.
- Roulin, A., Riols, C., Dijkstra, C., & Ducrest, A.-L. (2001). Female plumage spottiness signals parasite resistance in the barn owl (*Tyto alba*). *Behavioral Ecology*, 12(1), 103-110.
- Røskaft, E., Järvi, T., Bakken, M., Bech, C., & Reinertsen, R. (1986). The relationship between social status and resting metabolic rate in great tits (*Parus major*) and pied flycatchers (*Ficedula hypoleuca*). *Animal Behaviour*, 34(3), 838-842.
- Santostefano, F., Fanson, K. V., Endler, J. A., & Biro, P. A. (2019). Behavioral, energetic, and color trait integration in male guppies: testing the melanocortin hypothesis. *Behavioral Ecology*, 30(6), 1539-1547.
- Silva, J. E. (2006). Thermogenic mechanisms and their hormonal regulation. *Physiological reviews*, 86(2), 435-464.
- Stasiak, K., Kondracki, S., & Iwanina, M. (2019). Assessment of chosen semen characteristics of two colour morphs of the Arctic fox *Alopex lagopus* L. *Animal Science Journal*, 90(9), 1120-1126.
- Thierry, A. M., De Bouillane De Lacoste, N., Ulvund, K., Andersen, R., MeÅs, R., Eide, N. E., & Landa, A. (2020). Use of supplementary feeding dispensers by Arctic foxes in Norway. *The Journal of Wildlife Management*, 84(4), 622-635.
- Tietgen, L., Hagen, I. J., Kleven, O., Bernardi, C. D., Kvalnes, T., Norén, K., Hasselgren, M., Wallén, J. F., Angerbjörn, A., & Landa, A. (2021). Fur colour in the Arctic fox: genetic architecture and consequences for fitness. *Proceedings of the Royal Society B*, 288(1959), 20211452.
- Timisjärvi, J., Nieminen, M., & Sippola, A.-L. (1984). The structure and insulation properties of the reindeer fur. *Comparative Biochemistry and physiology. A, Comparative Physiology*, 79(4), 601-609.

- Tomasi, T. E. (1991). Utilization rates of thyroid hormones in mammals. *Comparative Biochemistry and Physiology Part A: Physiology*, 100(3), 503-516.
- Trullas, S. C., van Wyk, J. H., & Spotila, J. R. (2007). Thermal melanism in ectotherms. *Journal of Thermal Biology*, 32(5), 235-245.
- Underwood, L. S., & Reynolds, P. (1980). Photoperiod and fur lengths in the Arctic fox (*Alopex lagopus* L.). *International Journal of Biometeorology*, 24, 39-48.
- Våge, D. I., Fuglei, E., Snipstad, K., Beheim, J., Landsem, V. M., & Klungland, H. (2005). Two cysteine substitutions in the MC1R generate the blue variant of the arctic fox (*Alopex lagopus*) and prevent expression of the white winter coat. *Peptides*, 26(10), 1814-1817.
- Walsberg, G. E. (1991). Thermal effects of seasonal coat change in three subarctic mammals. *Journal of Thermal Biology*, 16(5), 291-296.
- Warret Rodrigues, C., & Roth, J. D. (2023). Feast to famine: Sympatric predators respond differently to seasonal prey scarcity on the low Arctic tundra. *Ecology and Evolution*, 13(3), e9951.
- West, P. M., & Packer, C. (2002). Sexual selection, temperature, and the lion's mane. *Science*, 297(5585), 1339-1343.
- Wingfield, J. C., & Romero, L. M. (2010). Adrenocortical responses to stress and their modulation in free-living vertebrates. *Comprehensive physiology*, 211-234.
- Zoeller, R. T., Tan, S. W., & Tyl, R. W. (2007). General background on the hypothalamic-pituitary-thyroid (HPT) axis. *Critical reviews in toxicology*, 37(1-2), 11-53.

