Phenotypic variation in past and present Scandinavian wolves (*Canis lupus* L.)

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Acknowledgements

Abstract

During the last century, the grey wolf (*Canis lupus* L.) was subject to extensive hunting pressure, which led to functional extinction of this species from the Scandinavian Peninsula around 1970. Wolves reappeared in Scandinavia in 1983 when one male and one female wolf established a territory at the border between southern Sweden and Norway. One new male contributed to the population in 1991, and these three individuals are considered the functional founders of the present wolf population in Scandinavia. The extant wolf population has been subject to numerous studies of pedigree, genetic variability and dispersal. Systematic documentation and studies of morphology have been called for, but are scarce. Also, few studies have investigated morphological differences between the extinct and extant Scandinavian wolf population. Available material from both populations consist of skulls and preserved hides in museum collections. In this thesis, skull measurements and size of foreleg melanin patches were compared between the two populations. Further, standardized photographic documentation and anatomical measures were obtained from recently shot wolves (n=35) during licensed hunts in Scandinavia in 2017 and 2018. This material was used to achieve redundant control for possible confounding factors in the museum material regarding size of melanin markings. The extinct wolf population (museum material, n=12) carried significantly larger foreleg markings than the extant population (n=72) overall. There were no significant effects of sex in either field or museum specimens, but in the field material juveniles had significantly larger melanin patches than adults. Frequencies of males vs juveniles were equally distributed between the extant and extinct fractions of the museum material. The field data further indicated that leg length did not affect the size of foreleg marks. A principal component analysis was performed on 16 craniometric variables obtained from adult males from the extinct (n=11) and extant (n=47) population. Separation of the populations was indicated for the 1st and 3rd PC axis. The most contributing variables to PC1 and PC3 was Condylobasal length (CbL) and forehead slope (Fs), respectively. The study populations significantly differed in both these variables, indicating a longer and flatter skull in extant compared to extinct wolves. Possible explanations for the observed morphological differences could be variation in geographical origin, adaptation to different environments, differences in available nutrients, or simply random founder effects followed by inbreeding. Further research should be carried out in order to understand the genotype-phenotype interaction underlying the differences in morphology between past and present wolves.

Contents

1 Introduction

1.1 Morphological plasticity in Canids

Presently represented by over 10 genera and more than 30 species, the Canidae are both the most widely distributed family of carnivores and one of the earliest originating extant group of this order. Canids and their biology fascinate both specialists and laymen, due to a range of attributes from their physical and cognitive abilities to their often highly complex and structured social behavior. Undoubtedly, our relationship with domestic dogs which probably dates back more than 14.000 years (Driscoll et al., 2009) contributes to this allure. Conjointly intriguing is the inherent capacity of many canids to generate both heritable phenotypic variation ("evolvability") and rapid responses to the environment. (Radinsky, 1973; Fondon & Garner, 2004; Van Valkenburgh, 2007; Curtis et al., 2018)

Like domestic dogs, wild canids exhibit great morphological variation, from the small fennec fox (*Vulpes zerda, shoulder height: 15-20 cm),* to the long-legged maned wolf (*Chrysocyon brachyurus, shoulder height: 74-91 cm)* (Castelló, 2018). Morphological variation reflects the variety in predatory strategies and dietary preferences found in the Canidae family (Slater et al., 2009). The diet of canid species can vary from hypercarnivores, omnivores, where half the diet consist of fruit, to insectivores like the bat-eared fox (*Otocyon megalotis megalotis)* (Slater et al., 2009; Asahara, 2013). One feature that distinguishes the family of Canidae from the rest of the carnivoran families is that they show higher flexibility and evolvability particularly in skull traits, such as higher flexibility in facial length and nasal length (Machado et al., 2018). Even though canids exhibit a wide range of predatory strategies, prey apprehension mostly rely on their teeth and skull (Slater et al., 2009). High flexibility and evolvability in these traits may allow canids to respond to selective pressure such as shifts in prey availability (Machado et al., 2018). Shorter snout and broader jaws are associated with greater bite force and the ability to take down large prey, while narrow and long faces are associated with fast-closing jaws, specialized in catching small animals (Slater et al., 2009).

The grey wolf (*Canis lupus*) was the first animal to become domesticated by humans, a process that started > 14,000 year ago, and resulted in the morphological diverse species known as man's best friend, the domestic dog (*Canis lupus familiaris)* (Driscoll et al., 2009). Domestic dogs displays the greatest morphological diversity found among terrestrial mammalian species (Schoenebeck & Ostrander, 2013). Variation in body proportions and size in domestic dog exceed the variation found among the wild canids (Wayne, 1986), and the variation in domestic dogs skull morphology is comparable to skull variation found in the entire Carnivoran order (Drake & Klingenberg, 2010).

A study conducted by Laidlaw et al. (2007) looked at the tandem repeat length variation in the genome of dogs and other carnivoran clades as a source of morphological variation. These authors found that wild and domestic canids possess an elevated occurrence of pure repetitive sequences that surpass those of other carnivoran clades (Laidlaw et al., 2007). Furthermore, domestic dogs have a genome-wide high slippage mutation rate, a character derived from their ancestors before domestication which has been preserved in modern wild canids. This may explain the high and quickly developing phenotypic variation found in both wild and domestic canids (Laidlaw et al., 2007; Machado et al., 2018). This distinctive quality makes wolves (*Canis lupus)* an interesting species for studying morphological changes through biological events in the wild, such as genetic bottlenecks, geographical dispersal, and environmental change.

1.2   The grey wolf

The largest member of the wild canids is the Grey wolf (*Canis lupus*) (Castelló, 2018). Historically, wolves were once the most widely distributed terrestrial mammal found throughout the Northern hemisphere (Mech, 1995). They are great dispersers and inhabit a range of different habitats like arctic tundra, deserts, grassland, mountains, woodland and prairies where they primarily prey on ungulates (Mech & Boitani, 2003; Geffen et al., 2004). Wolves are today no longer the most widely distributed terrestrial mammal, as they suffered persecution by humans on a global scale (Fritts et al., 2003). With the expansion of human settlement, competition for wild ungulates and fragmentation of habitats, large predators encountered conflicts with humans and their livestock (Delibes, 1990; Breitenmoser, 1998). Together with the rapid development of firearms, wildlife traps and the use of poison over the last two centuries, large predators were pursued and killed in high numbers (Pimlott, 1973;

Delibes, 1990; Mech, 1995; Wabakken et al., 2001). Wolves were especially targeted, and many European countries set out to exterminate the species (Boitani, 1995; Fritts et al., 2003). As a consequence, wolf populations reached critical low numbers around 1930-1960 and disappeared from many European countries (Delibes, 1990; Mech, 1995). The remaining populations were fragmented and isolated in small groups until the species became protected (Boitani, 2003; Pilot et al., 2014). After 1980, wolf populations started to expand and recolonize (Boitani, 2003). Many of the European wolf populations experienced severe genetic and demographic bottlenecks as a consequence of persecution of the species; this includes the Italian population, the Scandinavian population and the eastern and western Balkan populations to mention some (Vila et al., 2003; Fabbri et al., 2007; Djan et al., 2014).

1.3   The grey wolf in Scandinavia: Why phenotypes matter

Scandinavia followed the same trend as the rest of Europe. In Norway, a law of eradication of predators was sanctioned in 1845, which included bounties for many of the large predators (Bjerve, 1978). Similar events took place in Sweden and wolves were actively hunted until the species became legally protected in 1966 in Sweden and 1972 in Norway (Haglund, 1973; Delibes, 1990; Wabakken et al., 2001). By this time only a few individuals were observed on the Scandinavian peninsula, and the population became functionally extinct (Haglund, 1973; Wabakken et al., 2001).

In 1980, a sighting of wolves was reported in Scandinavia and in 1983 two wolves successfully reproduced in Värmland, Sweden, and continued reproducing in the following years (Delibes, 1990; Wabakken et al., 2001). In 1991 a new non-related male successfully reproduced, followed by two new males in 2008 (Jansson et al., 2015). These five individuals, one female and four males, are the founders of the new population in Scandinavia, and the three first individuals contribute over 95% of the present gene pool (Jansson et al., 2015).

Today the Scandinavian wolf population is estimated to be around 400 individuals (2017-2018) mainly distributed in central Scandinavia with the majority of the population found in Sweden and one-quarter of the population found in Norway close to the Swedish border (Wabakken et al., 2018). The number of breeding pairs and the geographical distribution of their territories is today governmentally controlled by legal hunting with a collaboration between the countries (Wabakken et al., 2018). The newly immigrated wolves in Denmark

should not be viewed as part of the Scandinavian population as these individuals stem from immigrating wolves from Germany/Poland (Andersen et al., 2015) and no gene flow between the two populations occurs.

The extant Scandinavian wolf population is closely monitored, and the population's pedigree is almost complete (Åkesson et al., 2016). Many studies have been conducted on genetic variability in the Scandinavian population and the risk for inbreeding depression (Vila et al., 2003; Liberg et al., 2005; Åkesson et al., 2016; Kardos et al., 2018). A study concerning genomic consequences of inbreeding by Kardos et al. (2018) found that many individuals in the severe inbred Scandinavian population have entire or nearly entire chromosomes that are homozygous. This study concluded that further studies should investigate the phenotypic consequences for the inbred population. To my knowledge only two such studies have been carried out, one investigating fluctuating asymmetry in skull traits of the extinct and extant populations (Wiig & Bachmann, 2014), and one examining malformations of the backbone in wolves from Finland and the extinct and extant Scandinavian populations (Räikkönen et al., 2006). Only the latter found indications of phenotypic consequences of inbreeding depression in the extant population. With these notable exceptions, compared to the extensive research on wolves and all aspects of their biology elsewhere (especially in North-America) (Musiani et al., 2007; Wheeldon & Patterson, 2012; O'Keefe et al., 2013), systematic registration of phenotypic variation in both the extant and extinct Scandinavian wolves can only be described as profoundly lacking. In an attempt to initiate more integrative studies, this thesis aims to further investigate and document morphological traits in the extant Scandinavian wolf population and compare trait variability in the extant population to the historical extinct Scandinavian wolf population.

1.4   A note on available study material

With the time and financial resources available to this MSc thesis, available study material was largely limited to collections at the natural history museums of Oslo and Stockholm. These collections consist of skulls and (from a lower number of individuals) preserved hides obtained in the period from 1830 - 2018. Hence, skull morphology and to a limited degree coat color variation can be compared between the study populations. In addition, I was able to obtain standardized photographic documentation and anatomical measures from recently shot wolves (whole bodies) during licensed hunts in Norway and Sweden during the winters of

2017 and 2018. This material is used to achieve redundant control for a possible confounding factor in the museum material, namely the lack of information about actual body size and its possible relationship to the size of melanin-based color patches (see below). While the ultimate goal would be to conduct studies linking genotype and phenotype, here I am limited to analyze and present only phenotypic variability in the study material.

1.5   Color variation, with a particular note on melanin patches

Variation in animal pigmentation has spurred and provided key models some of the most active and controversial fields in evolutionary biology including sexual selection, aposematism, crypsis and mimicry (Hamilton & Zuk, 1982; Majerus, 1998; McGraw & Hill, 2000; Sherratt & Beatty, 2003; Hoekstra, 2006; Gray & McKinnon, 2007) Furthermore, color polymorphisms provide the sort of raw material widely thought to facilitate sympatric speciation (Sinervo & Svensson, 2002; Bolnick & Fitzpatrick, 2007; Gray & McKinnon, 2007; Fitzpatrick et al., 2009). In vertebrates, two main pigment groups, carotenoids and melanins, cause color variation. Variation in carotenoid pigmentation is mostly condition-dependent and is assumed to signal individual "quality", in the sense that carotenoids are in limited supply and also have other critical functions (mainly in antioxidative processes and immune responses) than ornamentation (von Schantz et al., 1999; Clotfelter et al., 2007; Vinkler & Albrecht, 2010). In contrast, stable (and often heritable) color polymorphisms most often involve genes responsible for synthesis and deposition of melanin – in vertebrates the black pigment eumelanin, and red to brownish phaeomelanin (Alexandre Roulin, 2004; McKinnon & Pierotti, 2010).

Grey wolves can possess a range of different color morphs, from mono-colored fur in black or white to color pallets in grey, ochre, tawny and brown (Pocock, 1935). The most common fur morph is a variation of grey coat color and is common for the Eurasian subspecies, which the Scandinavian population is considered a part of (Pocock, 1935; Sillero-Zubiri et al., 2004). One striking coat color feature described in many of the subspecies of grey wolfs is a black stripe running down the front of the foreleg (Pocock, 1935; Ognev, 1962; Castelló, 2018). Due to technical difficulties with quantifying hue and intensity of other colors, I constrained the efforts within this thesis to develop a method to capture variation in this melanin-based marking.

I include these data since melanin-based coloration have also been associated with other phenotypic traits and the existence of behavioral syndromes in several vertebrates. Typically, individuals with pronounced melanin markings are found to be generally more aggressive, sexually active and resistant to stress and infection (Ducrest et al., 2008; Kittilsen et al., 2009; Kittilsen et al., 2012). Notably, in those species where dark hues or patterns are not used for camouflage, contrast-rich black patches are often major characteristics of life-history strategy, and are presumed to act as a social signal (Järvi & Bakken, 1984; Horth, 2003; Alexandre Roulin et al., 2008). Well documented examples are the breast stripe of the male great tit (*Parus major*), which is wider in socially dominant individuals, and the lion's (*Panthera leo*) mane, which is darker in successful pride leading males.

In some cases, such as in the lions, this co-variation is highly dependent on environmental and nutritional factors (West & Packer, 2002). However, there are also intriguing instances of strong genetic connections between melanism and personality traits such as proactive and reactive stress-coping styles (Ducrest et al., 2008; Kittilsen et al., 2009). In these cases, genetic variation controlling melanisation must also be controlling major physiological and behavioral features of the organism. This can occur through various types of linkage (Kirkpatrick et al., 2002; Kirkpatrick & Barton, 2006) or by genetic pleiotropy (Ducrest et al., 2008; Razeto-Barry et al., 2011; Khan et al., 2016). For instance, Khan et al. (2016) recently showed that the likely causal link between pronounced melanin spots in salmonid fishes and enhanced stress- and disease resistance depends on the steroid "stress hormone" cortisol stimulating expression of agouti signaling protein, an agonist of eumelanin synthesis. Cortisol production, in turn, is a highly variable and partly heritable trait in both teleost fishes and mammals (Koolhaas et al., 1999; Øverli et al., 2007).

In the case of wolves, the foreleg melanin stripe thus constitutes a phenotypic characteristic of conceivably high interest, which can potentially function as some sort of "quality" signal or serve as a phenotypic correlate of genetic heritage. In view of the above, I include as one achievable goal in this thesis to test whether this phenotypic trait differ between the extinct and extant populations of grey wolves in Scandinavia. Other general and specific aims are outlined below.

1.6   Aims and rationale

Despite the close monitoring of the extant Scandinavian wolf population, there is little standardized morphological documentation taken of wolves when killed. Such documentation is of potential value to future studies of pedigree, geographic origin, genotype-phenotype correlation, inbreeding depression, and bottleneck effects in comparison with phenotypic change. The current study therefore will develop a standardized methodology for taking photographs of *Canis lupus* in the field, and document phenotypes in the extant population by taking morphometric measurements while the animal is intact. Further, I aim to compare morphological traits between the extinct and extant Scandinavian wolf population. For the latter purpose, the available material from the two populations are preserved hides and skulls in museums collections in Scandinavia. In the present setting, this material can be used to investigate craniometric traits of skulls and melanin marks found on forelegs of wolf hides. These aims can be summarized as follows:

Aims of thesis:

- Develop a standardized method for photo collection and registration of visual and morphological characters of the extant wolf population in Scandinavia

- Compare selected morphological traits between the historical extinct wolf population and the extant wolf population in Scandinavia by using craniometric measures and foreleg melanin marks on preserved hides.

2   Material and Methods

2.1   Study material

2.1.1 Field work

Collection of measurements and photographs of wolf bodies and coats took place during the winters of 2017 and 2018. A total of 36 individuals (males: 20, females: 15, age range 0.7 – 9 years) were measured and photographed. All specimens were measured and photographed while in winter coat from October to March 2017 and again in the same period in 2018. During January 2017, field work efforts were concentrated on the licensed hunt in Sweden. Contact with the hunting parties in 3 territories (Blyberget, Loka and Brattfors) were established. The hunting teams allowed us access to specimens within 24 hours of death. A total of 12 wolves were measured and photographed in the course of this field work. Similarly, during January 2018, a total of 10 wolves were sampled from the wolfpacks Julussa and Osdalen during the licensed hunt in Norway. Additionally, one wolf was measured and photographed from the licensed hunt in Sweden 2018. Five dispersing wolves were made available for measuring and photographing by the National Veterinary Institute, SVA, in Uppsala, Sweden. Another eight individuals were made available for data collection by individual hunters outside the designated wolf protection area in Norway. Note that this material is a subsample of wolves shot during the study period, in some cases we were unable to be on site and take measurements before skinning and preservations of hides had to be initiated.

2.1.2 Museum collections

Wolf hides and skulls were made available for photography and external measurements by the Natural History Museum in Oslo and the Swedish Museum of Natural History in Stockholm. A total of 86 hides were photographed and 184 Scandinavian wolf skulls were measured.

Wolves reach adult size when they are about 12-14 months of age (Kreeger, 2010), and at an age of about 24 months the major dimensions of skull is fully developed to maximum size (Skeel & Carbyn, 1977; Nowak, 1979). Thus, since absolute age was not available for all specimens, for this study wolves were primarily separated in to two life stages; adults and juveniles. Individuals less than two years of age were classified as juveniles, and individuals of two years of age and older were classified as adults. Notably, within these categories individual variation in morphology does occur, and is often dependent on nutrient intake (Kreeger, 2010). Information regarding specimens age at death was obtained from the museums and Rovbase, a database and management tool that accumulates and verifies information regarding carnivores in Norway and Sweden ("Rovbase, Miljødirektoratet and Naturvårdsverket," 2018). Total numbers of specimens, sex, and life stage in all categories of the study material is summarized in table 1. below. Of note, due to the scarcity of adult females (n = 1) and relatively few juveniles (n = 4) available from the extinct population, analysis of craniometrics data was restricted to adult males.

Table 1. Number of samples obtained from the extinct and extant Scandinavian population of grey wolf

	Total	Adult Males	Adult Females	Juvenile Males	Juvenile Females	Missing information on sex or stage
Skulls, Extinct pop.	32	11	1	2	2	16
Skulls, Extant pop.	152	47	24	42	30	9
Hides, Extinct pop.	12	4	2	1	3	2
Hides, Extant pop.	72	21	10	20	17	4
Photographs, intact body	35	9	7	10	9	0
Measurements, intact body	35	9	7	10	9	0

2.2 Data collection

2.2.1 Photography

In the field, photographs were obtained from each specimen before measurements were taken, in order to minimize bloodstains on the fur. All photographs were taken with a Canon EOS 60D with a Sigma DC 17-50mm 1:2.8 EX HSM lens, using manual camera setting adjusted to every photo shoot. All photos were shot and saved in RAW format. White balance was set manually in the camera by photographing a white balance card (Lastolite, LL LR1250) and choosing the picture as a reference for the camera to create the white balance profile. The camera settings stayed the same throughout every photo shoot. The photographing was done with hand held camera as the objects were large and field conditions could be constraining. Shooting large objects with a tripod demands extensive equipment and longer rigging time, exceeding the narrow time limit with access to the animals. The downside of shooting with a hand-held camera in poor light is the constraints it puts on exposure time, which again limits the focal length of the camera. When possible, extra light sources were applied, and placed in an angle that minimized the amount of shadow on the object. An X-rite Color Checker Passport (X-rite Inc. model no. MSCCPP) was photographed together with the ID of the specimen in each photo series, in order to correct color in photos to true colors with the Color Checker Camera Calibration software. For accurate results in the Color Checker Camera Calibration software, the camera settings and light conditions were the same for all the photos taken of each individual. A white plastic sheet displaying a 10x10 cm grid was used as a background for the photos (see figure 1). Each wolf was photographed from different angles, capturing the whole body of the wolf in lateral, dorsal, and ventral views. Close-up photos were taken of the wolfs head in lateral and dorsal view, paws, front and back legs, tail, teeth, ears, and undercoat fur. A total of 36 wolves were photographed, numbers, sex and life stage of these are summarized in table 1.

A similar procedure was applied for photographing wolf hides. The hides were laid as flat as possible on the background sheet and photographed, capturing the whole hide (see figure 1). Closeup photographs were taken of the tail, head, cheek, forelegs and undercoat fur. The background sheet was made visible in the photographs of the forelegs, making it possible to use the grid as a measure of length later in the data processing. A total of 84 hides were

photographed, 12 from the extinct population (year range 1830-1979) and 72 from the extant (year range 1984-2016), for summary of sex and life stage see also table 1.

Figure 1. Photograph of a hide (Museum no. 20115040, NRM, Stockholm) with background sheet and color checker passport

2.2.2 Field morphometry

Morphological measurements were obtained from wolf bodies in the field. The collection of measurements was carried out as fast as possible after the wolfs death, usually within 48 hours of death and before skinning of the hide. The specimens ID, age, sex, weight, coordinates of capture and date of capture were noted. A total of 38 measurements were taken on each specimen (figure 2, table 2), by using a measuring tape and vernier caliper. Measurements were done to the nearest 0.1cm. Specimens were measured while lying down on the side. When body length was measured, the wolfs neck was straightened out so the head was linear with the shoulder. When limbs were measured, the limbs were held in a position mimicking a wolf standing in an upright position. A total of 36 wolves were measured, see table 1 for more detail. Measurements were only taken once due to the material only being available for a restricted time period. The repeatability of these measurements should be addressed in future studies.
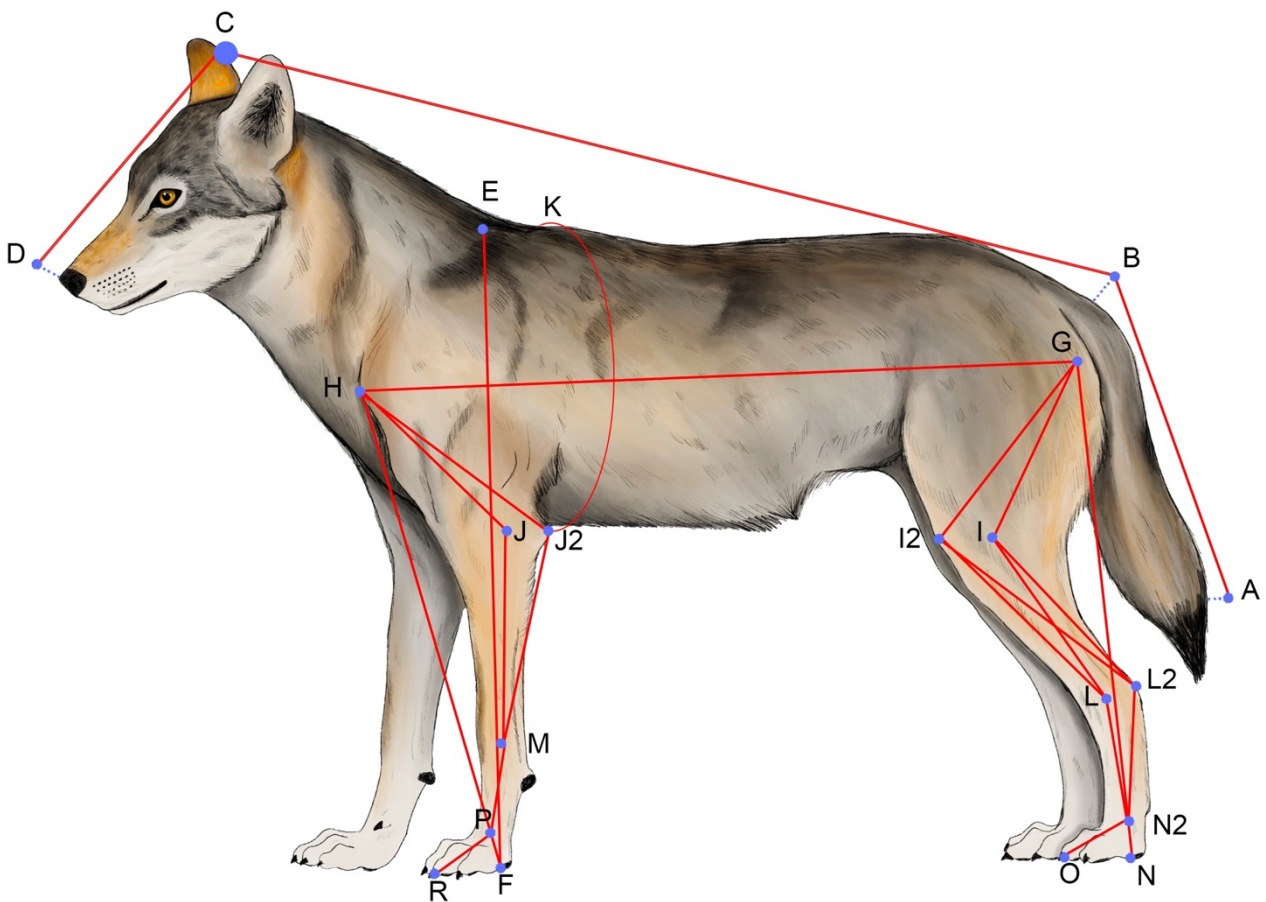

Figure 2. Illustration of a grey wolf and definition of body measurements.

Table 2. Body measurements and description

	Body measurement	Explanation
A-B	Tail length	From the base of the tail to the end of the vertebrae
B-C	Body length	From the base of the tail to occiput
C-D	Head length	From occiput to the tip of the nose
E-F	Height at withers	From the upper extremity of the scapula to metacarpal pad
G-H	Rump to shoulder	From the upper extremity of the femur to the upper extremity of the humerus
G-N	Rump to rear foot	From the upper extremity of the femur to the metatarsal pad
G-I	Thigh length	From the upper extremity of the femur to the lower extremity
G-I2	Thigh length 2	From the upper extremity of the femur to the patella
I-L	Shank bone length	From femur lower extremity to the lower extremity tibia
I_L2	Shank bone length 2	From femur lower extremity to the calcaneum
I2-L	Shank bone length 3	From the patella to the lower extremity tibia
I2-L2	Shank bone length 4	From femur lower extremity to the calcaneum
L-N2	Rear foot length	From the lower extremity of tibia to the end of the metatarsals
L2-N2	Rear foot length 2	From the calcaneum to the end of the metatarsals
N2-O	Rear paw length	From the upper extremity of the metatarsals to the outer edge of the digital pad
OR1	Rear paw length 2	From metatarsal pad to outer edge of the digital pad
OR2	Rear pad width	Greatest width of paw when stretched out, from toe to toe
H-F	Shoulder to foot length	From the upper extremity of the humerus to the metacarpal pad
H-J	Arm length	From the upper extremity of the humerus to the lower extremity of the humerus
H-J2	Arm length 2	From the upper extremity of the humerus to the olecranon
J-M	Forearm length	From the lower extremity of the humerus to the carpus
J2-M	Forearm length 2	From the olecranon to the carpus
M-P	Front foot length	From the carpus to the end of metacarpus
P-R	Front paw length	From the end of the metacarpus to the outer edge of the digital pad
OF1	Front paw length 2	From the metacarpal pad to the edge of the digital pad
OF2	Front paw width	Greatest width of paw when stretched out, from toe to toe
K	Chest circumference	Circumference of the chests largest point.
W	Ear length	From the apex of the ear to the base of the ear
Q	Ear width	From the spine of helix to the cutaneous marginal pouch
Z	Length between ear	From the spine of helix left ear to the spine of helix right ear
ZyG	Zygomatic breadth	Breadth between the zygomatic arches
FsI	Forehead slope intact body	Place a straight ruler on the tip of the nose bone and to the top of the forehead. Measure depth of the slope at the base of the nose to the ruler
BuCI	Breadth of upper canines	5 mm from the tip of the canine, measure the breadth between canines' outer edges
BlC	Breadth of lower canines	5 mm from the tip of the canine, measure the breadth between canines' outer edges
LuC	Length of upper canines	Length of the right and left canine
LlC	Length of lower canines	Length of the right and left canine

2.2.3 Skulls morphometry

To describe skull characteristics, a total of 16 measurements were taken (figure 3, table 3). The measurements were chosen based on a craniometric study of Latvian wolves by Andersone and Ozolins (2000). Two additional measurements were taken, forehead slope and mandible length II. Skulls were made available by the Natural History Museum in Oslo and the Swedish Museum of Natural History (Stockholm). Measurements were taken with a 30 cm vernier caliper done to the nearest 0.01mm. Ideally, all measurements should be made twice, but due to time limitation this was not possible. However, to investigate reproducibility of the measurement protocol, three measurements, CbL, BaL and ZyB were repeated for 30 random specimens. A linear regression analysis was performed on the two sets of measurements, and the correlations were 99% for CbL ($R^2 = 0.99$, $F_{(28)} = 3275$, $p < 0.0001$), 98% for BaL (($R^2 = 0.98$, $F_{(28)} = 1535$, $p < 0.0001$), and 99% for ZyB ($R^2 = 0.99$, $F_{(28)} = 4632$, $p < 0.0001$), suggesting great reproducibility in the measurement protocol.
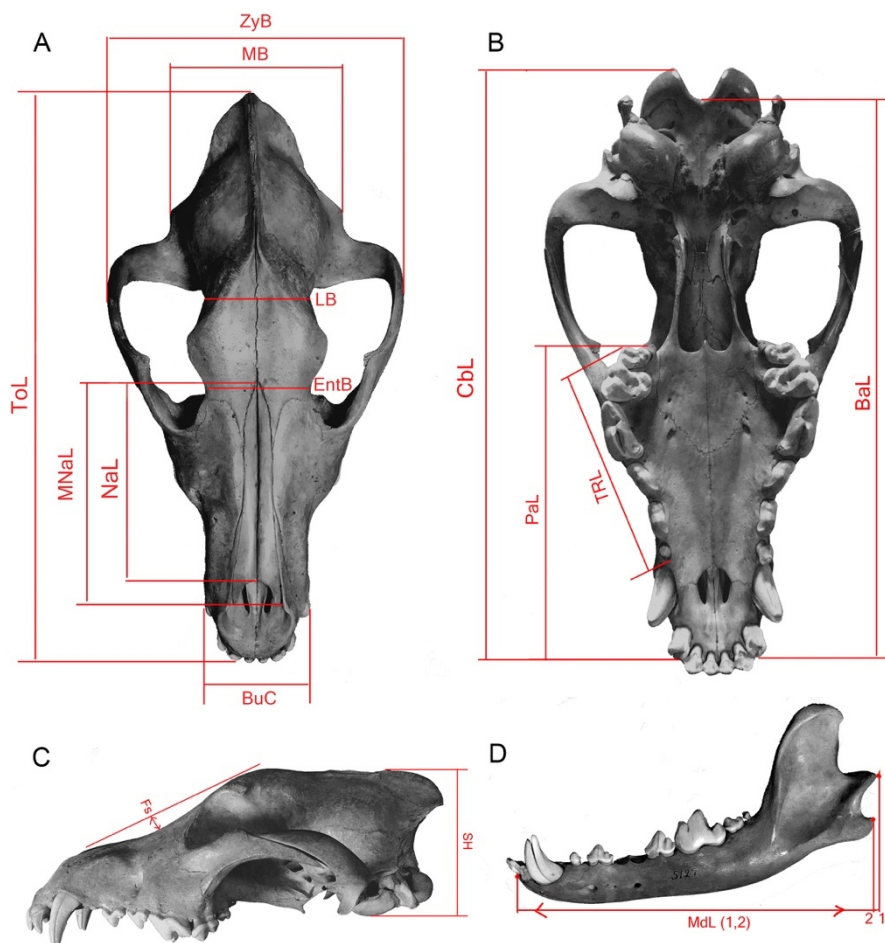

Figure 3. Definition of grey wolf skull measurements. **A**: dorsal view, **B**: ventral view, **C**: lateral view, **D**: lateral view of mandible. For abbreviation see table 3.

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Table 3. Skull measurements and abbreviations used in the morphometric analysis

ABBREVIATIONS	SKULL MEASUREMENTS	EXPLANATION
TOL	Total length	From the sagittal crest to the prosthion
MNAL	Maximum nasal length	Maximum nasal bone length
NAL	Nasal length	Minimum nasal bone length
ZYB	Zygomatic breadth	Breadth between outer edges of zygomatic arches
MB	Mastoid breadth	Breadth between mastoid process
LB	Minimum breadth of skull	Least frontal breadth
ENTB	Minimum breadth between orbits	Breadth of entorbitale
BUC	Breadth between outer edges of upper canine	Breadth between outer edges of upper canine
CBL	Condylobasal length	From condyloid process to the prosthion
BAL	Basilar length	From prosthion to the basion
PAL	Palate length	From prosthion to edge of palatinum
TRL	Length of upper tooth row	From anterior edge of P^1 to posterior edge of M^2
SH	Skull height	From the sagittal crest to the tympanic bulla
FS	Forehead slope	The slope of the forehead, using a straight ruler and placing it on the exterior end of the nasal bone and the forehead. Measuring the gap between the ruler and the nasal bone.
MDL I	Mandible length I	From infradentale to condyle process
MDL II	Mandible length II	From infradentale to angular process

A total of 184 skulls of Scandinavian grey wolf were measured, where 32 were from the extinct population and 152 were from the extant population, for summary of sex and life stage in the two populations see table 1. Some measurements could not be made due to damage of the skulls, these measurements were treated as missing values. Due to the highly skewed sex ratio and few juveniles available in the extinct population, only craniometrics data for adult males were used for comparison between the populations.

2.3 Photograph processing

2.3.1 Foreleg mark

Photographs of wolf hide forelegs were initially processed in Adobe Photoshop CC (19.1.6. Release). Standardizing an area of interest based on the individual size on hides is difficult due to various skinning techniques and lack of bones as standardizing points. Instead, a 10x30 cm area of the foreleg and surrounding area, including all black markings, was extracted from each image based on the background grid measurements and used as the area of interest for further calculations. A brush tool in similar color as the fur was used in the area of interest to remove holes, claws, background etc. that could affect the image analysis. Further processing was performed in Fiji, an open-source software project for image analysis, building on the ImageJ platform (Schindelin et al., 2012). Images were converted to 8-bit, before a threshold filter was applied. Threshold filter paints out dark contrast in images and was manually adjusted for each image to fit the black pigmented area in the fur, by using the original image as a reference. The software was used to find the number of pixels in the marked area relative to the total number of pixels in the image and was converted to cm^2. The outline of the marked area was manually verified against the original photograph. When possible, both legs were processed for each animal and the mean of the two legs were used in the analysis. Two observers, one blinded and one non-blinded did the image processing and analysis in Fiji. A linear regression analysis was performed on the results from the two observers. The correlation between the two observers was 92 % ($n = 84$, R$^2 = 0.92$, $F_{(82)} = 907$, $p < 0.0001$). Mean data from both observers were used in further analysis.

The same method of photo processing and analysis was applied for animals photographed in the field. Initial attempts to quantify hue and intensity of general coat coloration were aborted due to time constraints.

2.4　Statistical analysis

Statistical analysis was performed in R using RStudio 1.1.453 (RStudio Team, 2016) and visualized with the R package ggplot2 (Wickham, 2016).

2.4.1　Craniometrics

I first performed an exploratory Principal component analysis (PCA) to determine which variables contributed the most to variance within the data set. Principal component analysis is a multivariate statistical technique which simplifies large data matrices with related variables. This is done using orthogonal transformation to extract the dominant pattern and estimate correlation structure of variables in the data matrix, creating a new set of variables called principal components (PCs) (Wold et al., 1987; Abdi & Williams, 2010). The outcome of the PCA can be visualized graphically by using the variables or individuals factor scores as coordinates in the components space (Abdi & Williams, 2010).

Principal component analysis of craniometric measurements was performed using FactoMineR (Lê et al., 2008) and visualized with factoextra (Kassambara & Mundt, 2017). As PCAs require complete datasets an imputation was done using the missMDA package (Josse & Husson, 2016) to estimate parameters for an incomplete dataset using a principal component method (Josse & Husson, 2016). Only adult males were used in the PCA. Adult females were excluded from the analysis due to low sample size of females. Due to greater variation in juvenile skulls properties due to growth, combined with a low number of juveniles in the extinct population, juveniles were also excluded from the analysis. Consequently, only adult males were used in the PCA. The morphometric dataset used in the PCA thus contained 16 measurements from 58 adult males, 11 extinct and 47 extant. Principal component analysis requires linearity of the dataset, and deviation from linearity was not detected for any of the PC's or their most contributing (<5%) variables (Runs test, data not shown). To test for population differences in PC values and in the greatest contributing variable in differing PC's, a non-parametric Mann-Whitney U-test was used due to the difference in sample size.

2.4.2 Foreleg mark

Due to considerably unequal n in the two populations, data on foreleg melanin markings were compared between the extinct and extant population by non-parametric Mann-Whitney U-test. Effects of possibly confounding variables such as sex, season of capture, life stage and somatic length and length of the foreleg (known for field specimens) were assessed by GLM. Although these data met parametric criteria, this approach was not used to assess actual population differences due to it's possible sensitivity to unequal sample sizes (Keppel & Wickens, 2004). GLM on field data revealed that life stage (adult vs juvenile) but not leg length or length of the animal as such had a significant effect on melanised area. Life stage did not vary systematically between the extinct and extant population, so it is assumed that this possibly confounding variable did not affect the comparison between the populations mentioned above.

Foreleg length from the field data was calculated by adding forearm length (J-M) with the length of the front foot (M-P) and the front paw (P-R) (figure 2, table 2). A Shapiro-Wilk normality test was applied to test for normality of data, and a two-sample t-test was conducted to test for differences in foreleg length between juveniles ($n = 21$) and adult ($n = 16$) from the field dataset.

3 Results

3.1 Skull morphometry in in the extinct and extant Scandinavian wolf population

In the principal component analysis, the four first principle components each explained more than 5%, and together 85.2% of the total variation in the data set (Eigenvalues: PC1 = 9.87; PC2 = 1.73; PC3 = 1.10; PC4 = 0.93), (figure 4). Principal components explaining less than 5% of the variance were excluded from further analysis.
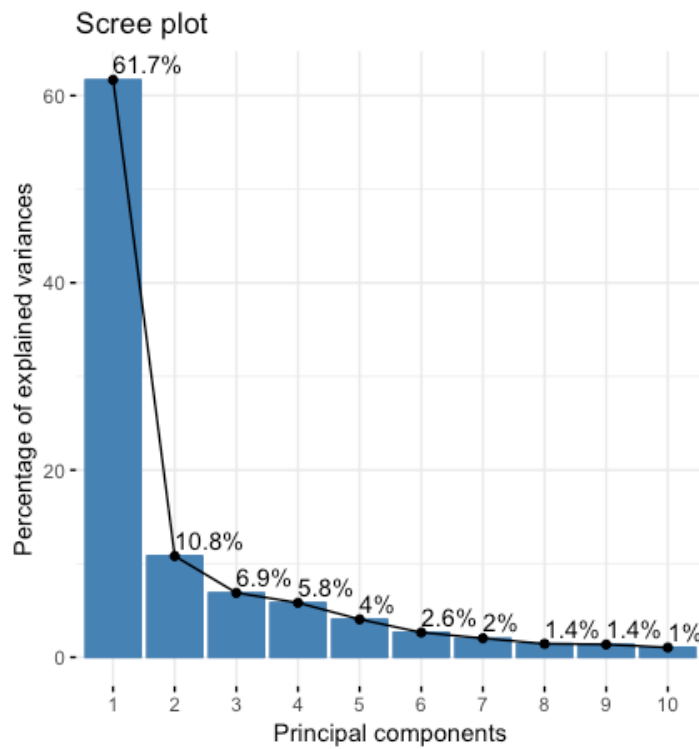

Figure 4. Scree plot over percentage of explained variance for ten principal components. PC1, 2, 3 and 4 together explained 85.2% of the total variation.

A correlation matrix plot (figure 5) illustrates the percentage each variable contributes to the principal components, showing that a majority of the variables greatly contribute to PC1. For PC1, ten variables are shown to range above the expected average contribution line at 6.25% (figure 6.A). The six first variables stand out with the greatest contribution to PC1. These variables, CbL, BaL, MdLII, MdLI, ToL and PaL (figure 3), are all measurements related to skull length. The next variables contributing to PC1 which are not related to measurements of length are MB and ZyB. These variables are measurements related to the width of the skull (figure 3). The correlation matrix (figure 5) illustrates that PC2, PC3, and PC4 have fewer variables that largely contribute to the components. Minimum width of skull (LB) and minimum width between orbits (EntB) are measurements related to forehead width. These two variables contributed most to PC2 (figure 6B), which explains 10.8% of the overall variation. Forehead slope (Fs) is the greatest contributor of the variables in PC3 (figure 6.C), which explains 6.9% of total variation. The variables with the greatest contribution to PC4, which explained 5.8% of total variation, are MNaL and NaL, measurements of nasal length, and BuC, which is the measurement of width between the upper canines (figure 6D).
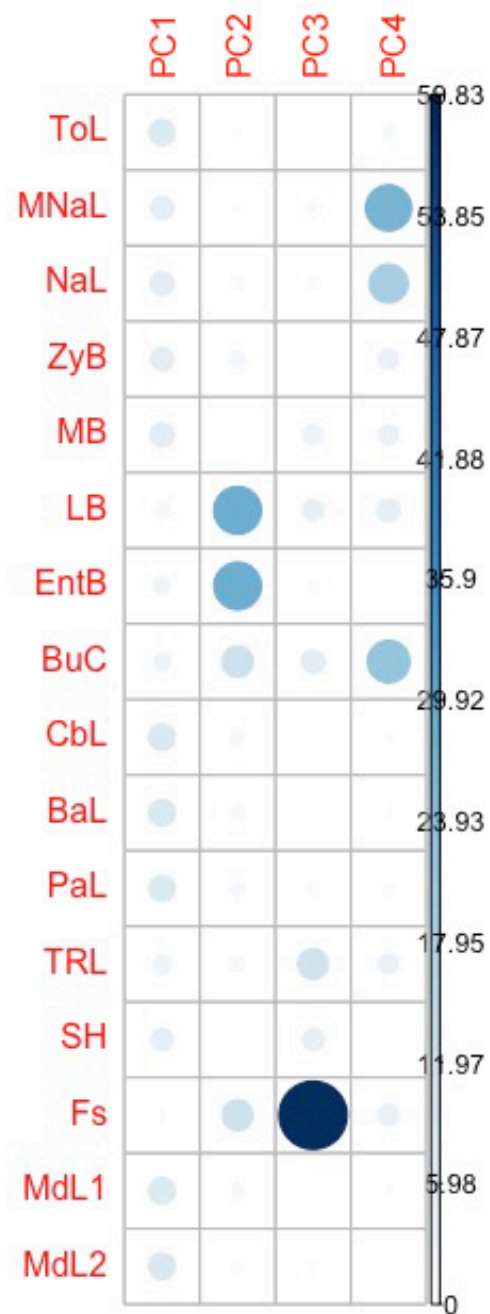
Figure 5. Correlation matrix plot displaying the percentage of contribution of variables for each principal component.
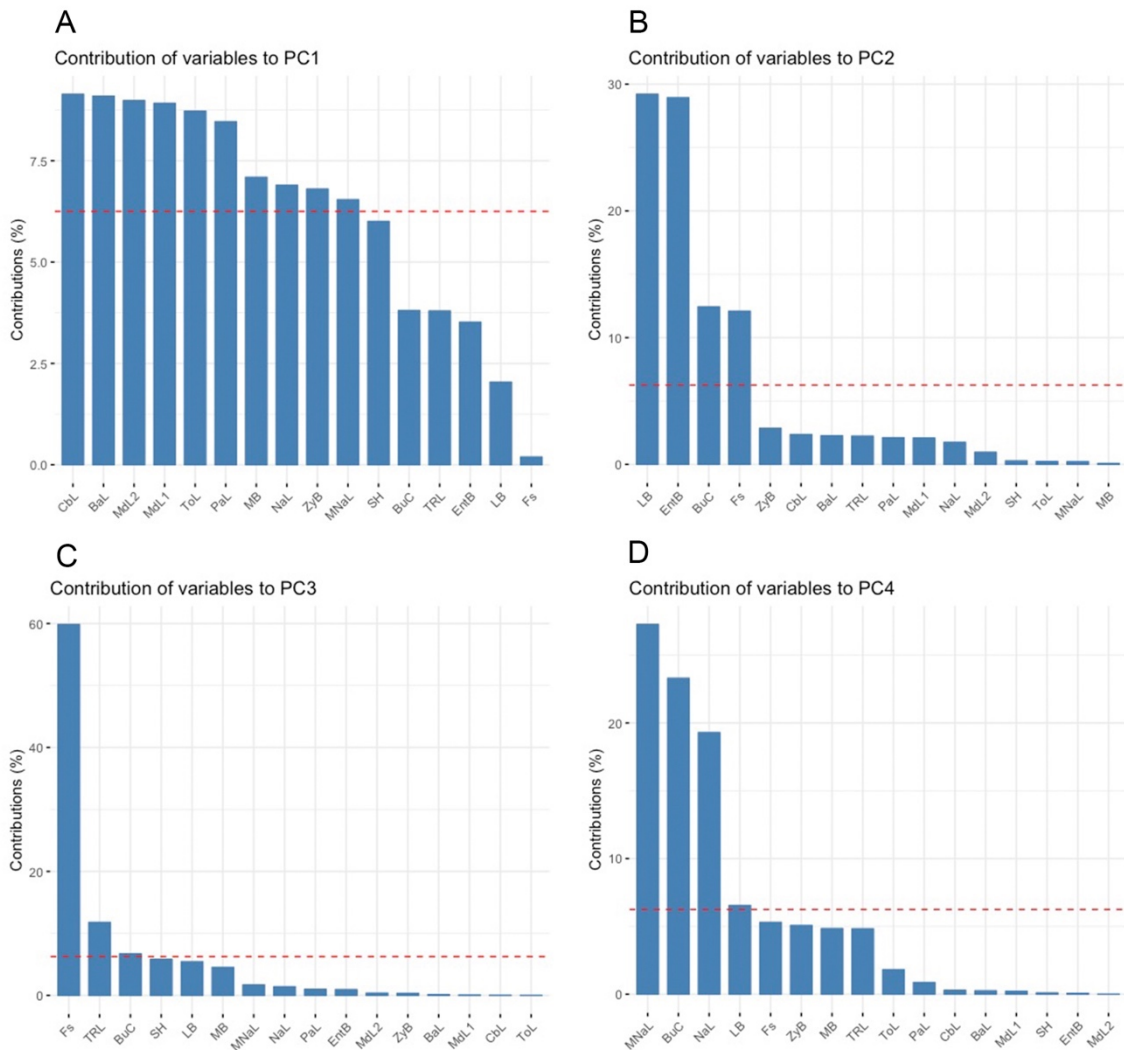
Figure 6. Bar plot illustrating the total contribution of variables to **A**: PC1, **B**: PC2, **C**: PC3, **D**: PC 4. Dashed red line indicates expected average contribution of variables to the principal components, at 6.25%.

A variable factor map of PC1 versus PC2 (figure 7A) shows that measurements representing length group together and highly contribute to the PC1 axis. EntB, LB, and BuC contribute to both component axes. The individual factor map of PC1 versus PC2 (figure 7.B) displays individual observations' PC values and a confidence ellipse illustrating the population mean for each population (extinct and extant). There is separation between the population mean placements on the PC1 axis, with the extinct population mean on the negative side of the axis, and the extant population mean on the positive side of the axis The extinct population's individual points are primarily to be found on the negative side of the PC1 axis with some

individuals on the positive side near zero. This suggests a clear separation of the populations by PC1 (i.e. mainly length measures) but not PC2 (i.e. mainly width measures).

A variable factor map of PC1 plotted against PC3 (figure 8A) shows that Fs is greatly contributing to PC3. The rest of the variables are grouped together and are highly contributing to PC1. The individual factor map of PC1 versus PC3 (figure 8B) shows a separation of the two populations on both component axes. The extinct population are primarily found on the positive side of the PC3 axis. PC1 plotted against PC4 did not indicate separation on the PC4 axis (see appendix, A2).

Figure 7. Variables factor map and individual factor map for PC1 and PC2

A: Variables factor map that shows correlation between the variables and the components. Gradient color displays the contribution of the variables to the PCA. The unit of the contribution gradient = [(C1 x Eig1) + (C2 x Eig2)] / (Eig1 + Eig2) (C = contribution of the variable on PC, Eig = Eigenvalues of PC). The vectors' length and placement project the loading of the variables in the analysis. Longer vectors represent greater contribution of the variable. Vectors direction represent variables relation to each other and the components, when grouped together, variables are positively correlated, when positioned on opposite sides variables are negatively correlated.

B: Individuals factor map that shows displays PC1 and PC2 values for individual animals. Ellipses illustrate groups mean placements. Individuals from the extinct population are displayed with red triangles. Individuals from the extant population are displayed with yellow circles
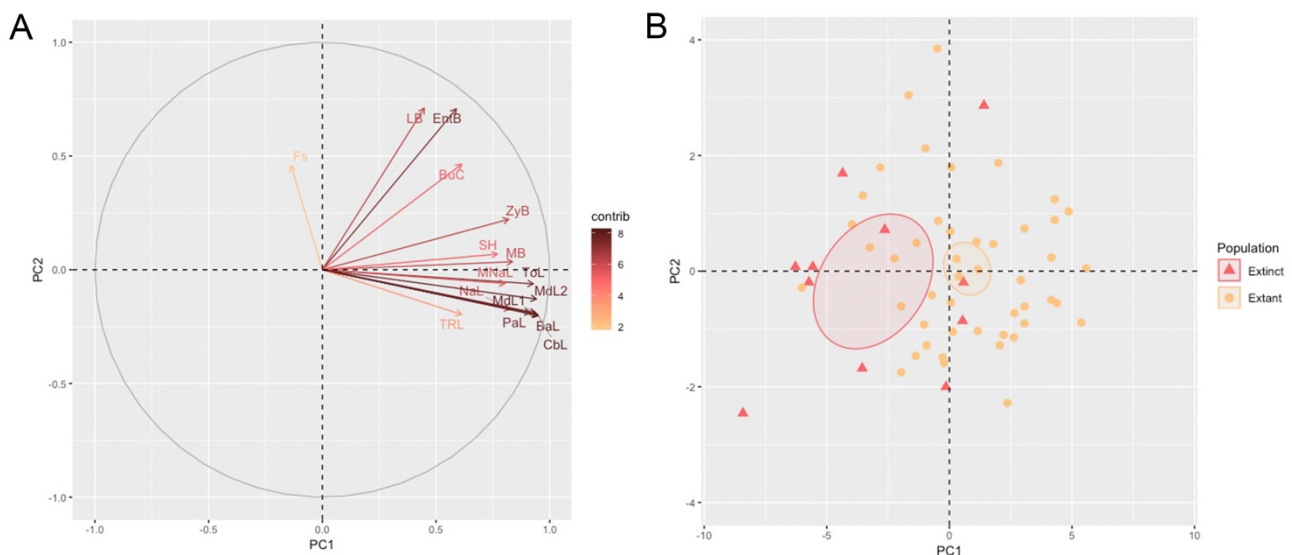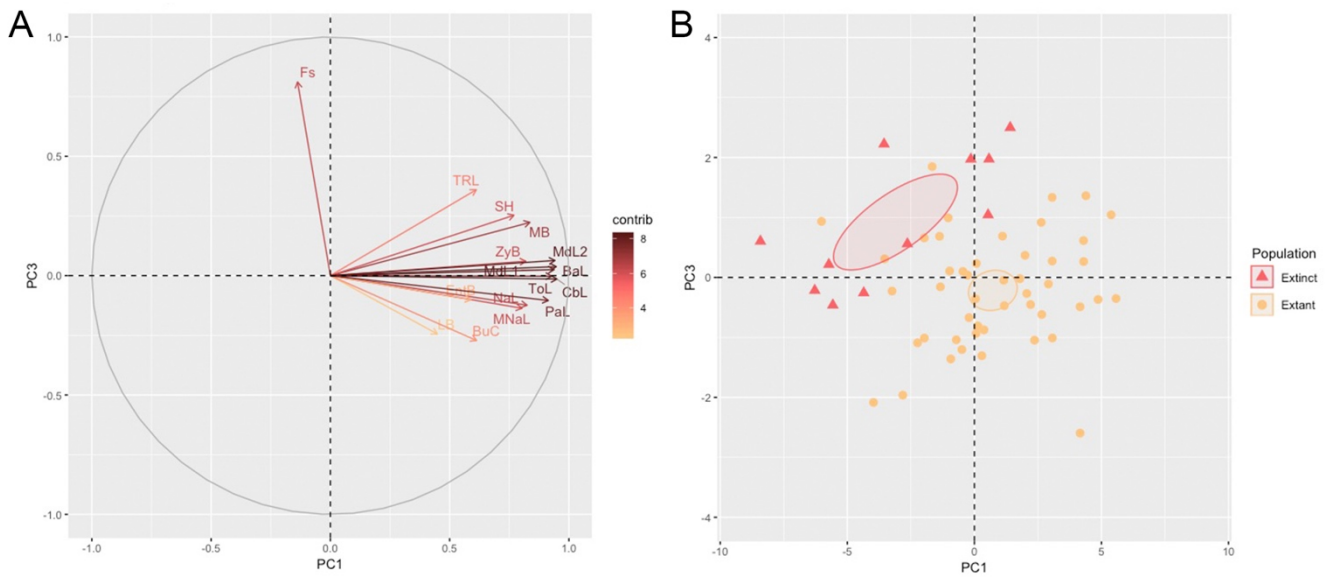
Figure 8. Variables factor map and individual factor map for PC1 and PC3

A: Variables factor map that shows correlation between the variables and the components. Gradient color displays the contribution of the variables to the PCA. The unit of the contribution gradient = [(C1 x Eig1) + (C2 x Eig2)] / (Eig1 + Eig2) (C = contribution of the variable on PC, Eig = Eigenvalues of PC). The vectors' length and placement project the loading of the variables in the analysis. Longer vectors represent greater contribution of the variable. Vectors direction represent variables relation to each other and the components, when grouped together, variables are positively correlated, when positioned on opposite sides variables are negatively correlated.

B: Individuals factor map that shows displays PC1 and PC3 values for individual animals. Ellipses illustrate groups mean placements. Individuals from the extinct population are displayed with red triangles. Individuals from the extant population are displayed with yellow circles

The individual PC scores were used to statistically test for significant differences between populations for each principal component. Due to low sample size in the extinct population, a non-parametric Mann-Whitney U-test was applied. There were significant differences between the populations in PC1 ($W = 410$, $p = 0.002$) and PC3 ($W = 118$, $p = 0.004$), (Figure 9). There were no significant difference between the populations in PC2 ($W = 284$, $p = 0.62$) or PC4 ($W = 209$, $p = 0.33$).

The variables with greatest contribution to PC1 and PC3 were also compared between the populations using a Mann-Whitney U-test. Significant differences were found between the two populations in Condylobasal length (CbL), ($W = 357$, $p = 0.004$), where the extant population had higher mean value than the extinct population (figure 10A). In the case of forehead slope (Fs), ($W = 61.5$, $p = 0.006$), the extinct population exhibit a greater mean slope than the extant population (figure 10.B). This suggest that the extant population has longer and flatter skulls than the extinct population.
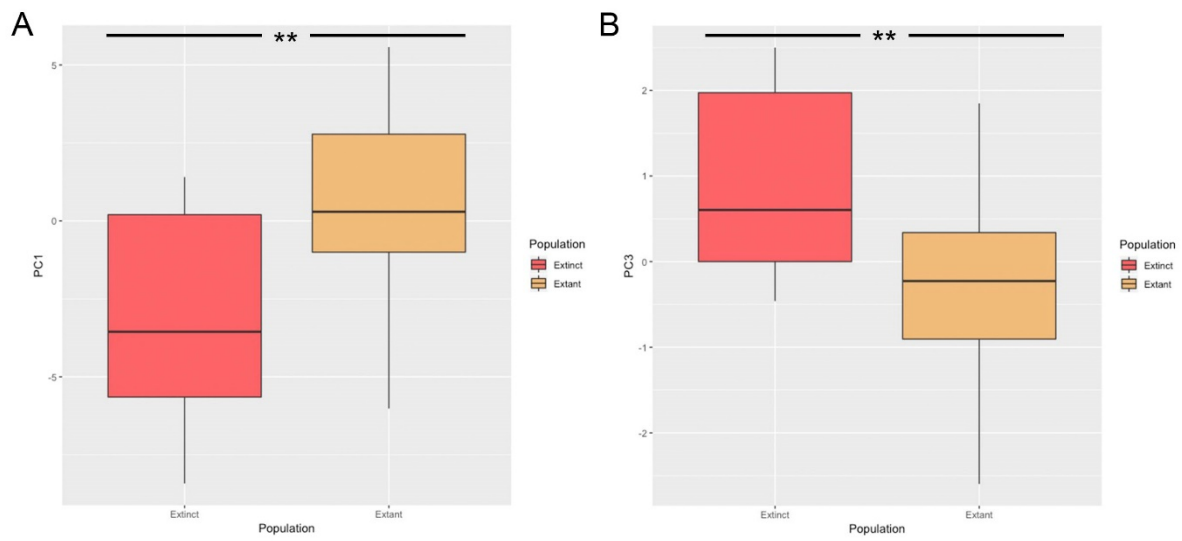
Figure 9. Boxplot comparing PC values between the populations. **A**: PC1, Mann-Whitney U: $W = 410$, $p = 0.002$, **B**: PC3, Mann-Whitney U: $W = 118$, $p = 0.004$
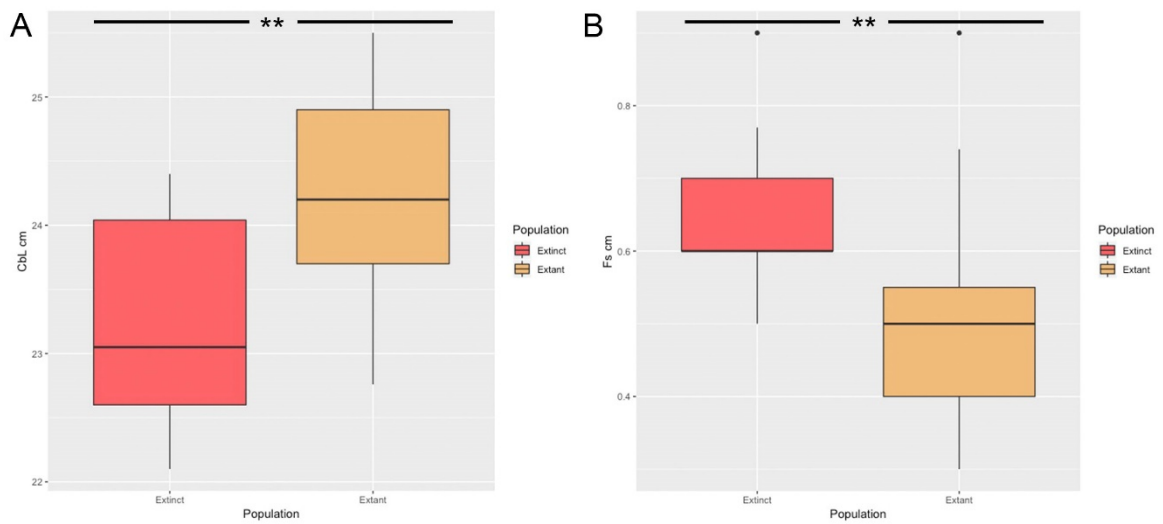
Figure 10. A: Condylobasal length (CbL) for the extinct and extant populations Mann-Whitney U: W = 357, p = 0.004 **B**: Measurement of forehead slope (Fs) for the two populations, Mann-Whitney U: W = 61.5, p = 0.006

Correlation between age and the measurements CbL and Fs was tested for each population using linear regression analysis. There were no significant effect of age on CbL in either the extinct or extant population (figure 11). Fs was not related to age in the extinct population (figure 12A), but weakly so in the more numerous specimens from extant population (R^2 = 0.12, p = 0.04, figure 12B). Correlation between age and the principal components were also tested, but not found to be present (data not shown).
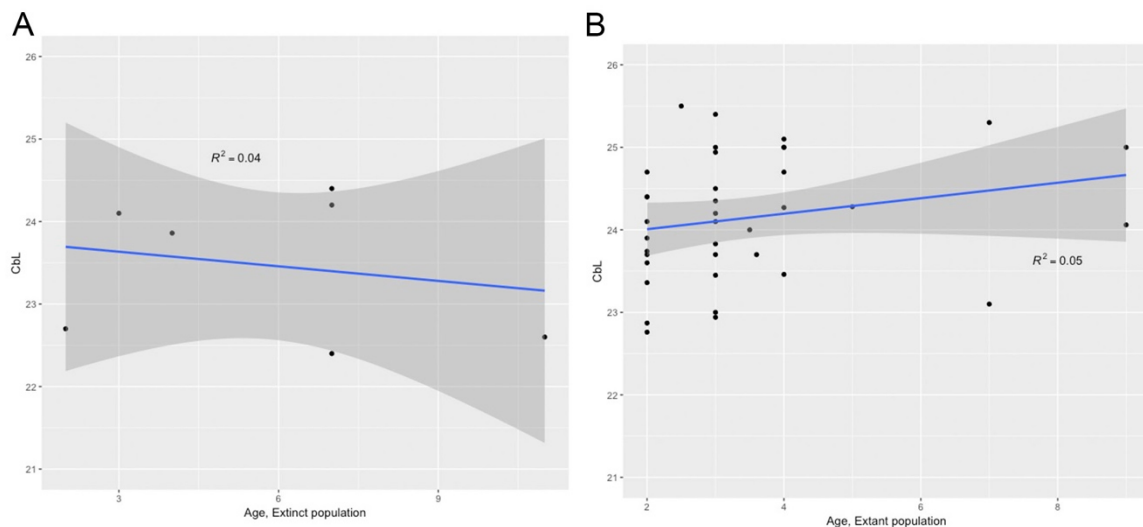
Figure 11. Linear regression between CbL and age. **A:** Extinct population, R^2 = 0.04, p = 0.64. **B:** Extant population, R^2 = 0.05, p = 0.18.
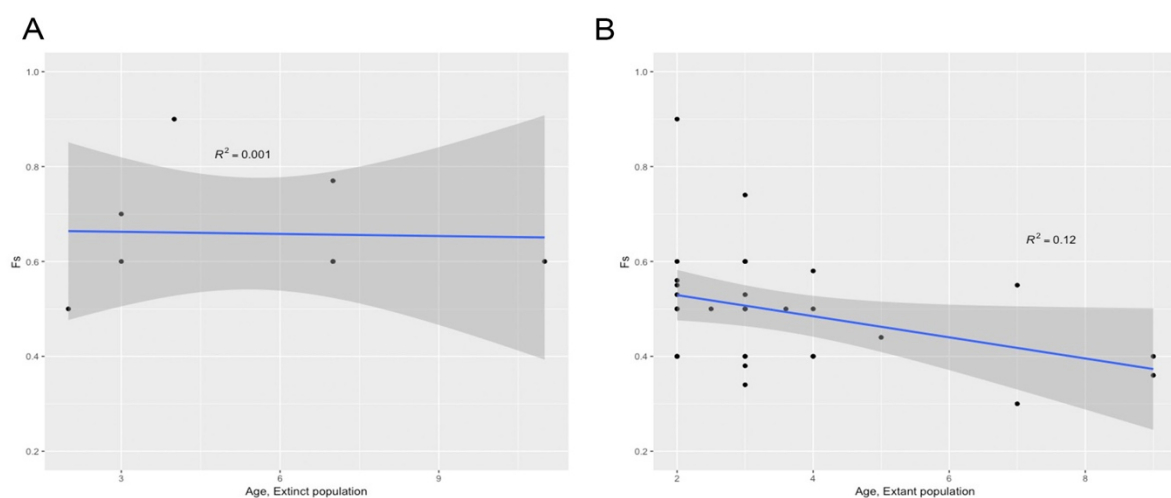
Figure 12. Linear regression between Fs and individual age. **A:** Extinct population, R^2 = 0.001, p = 0.93. **B:** Extant population, R^2 = 0.12, p = 0.04.

3.2 Foreleg mark size

GLM on field data revealed that life stage (adult vs juvenile) but not leg length or somatic length of the animal had a significant effect on melanised area. Numerically, juveniles showed larger melanin patches (mean ± SEM: 0.33 ± 0.08 cm^2) than adults (mean ± SEM: 0.15 ± 0.04 cm^2), but there was no effect of life stage on leg length (figure 13). An effect of life stage on melanin markings was not present in the extant museum material. Outcomes of the GLM are summarized in table 4, below. In those specimens where these variables had been registered, neither season or life stage varied systematically between the extant (summer:winter ratio: 21:51; adult:juvenile ratio: 31:37) and extinct (5:7; 6:4, respectively) populations. Hence, I proceeded to compare absolute values for foreleg mark size between the extant and extinct populations.
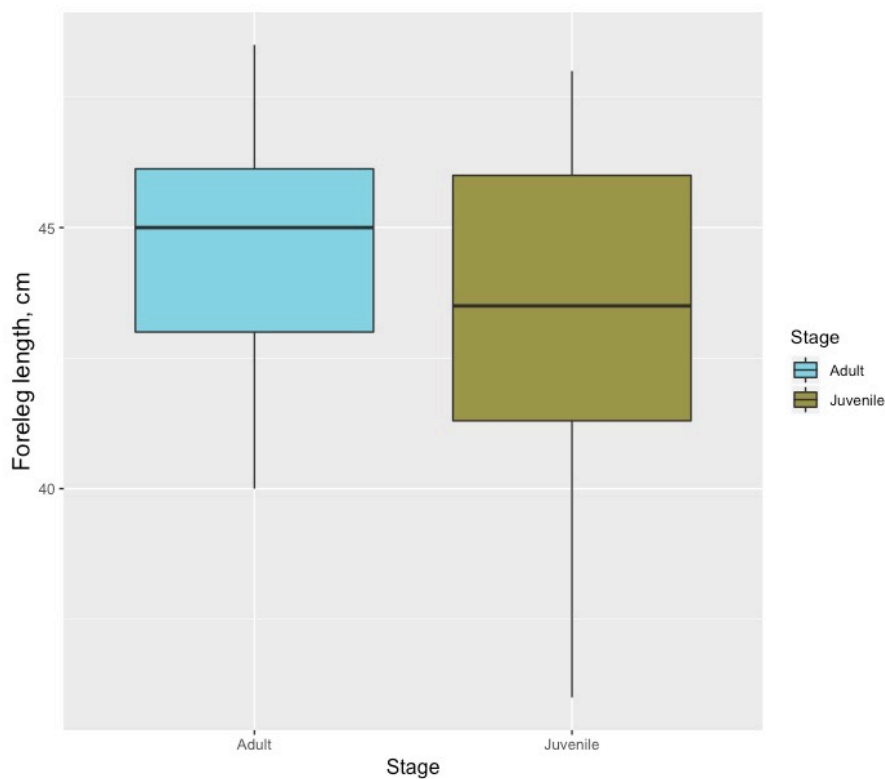
Figure 13. Foreleg length of adults and juveniles from the field dataset, t-test: $t_{(35)} = 1.39$, $p = 0.17$

Table 4. GLM effect tests for possible confounding variables affecting foreleg mark size.

Field data	DF	Chi Square	p
Sex	1	0.39	0.54
Life stage	1	4.78	**0.03**
Foreleg length	1	0.15	0.70
Somatic length	1	0.49	0.48

Museum specimens, extant population	DF	Chi Square	p
Sex	1	0.15	0.70
Life stage	1	0.43	0.50
Season	1	0.01	0.90

A non-parametric Mann-Whitney U-test was performed to investigate foreleg mark size differences between the extinct ($n = 12$) and extant ($n = 72$) populations. A significant difference in foreleg mark between the populations was found ($W = 31$, $p < 0.001$). As illustrated in figure 14, the extinct population carried significantly larger foreleg marks than the extant population (also, see representative examples in figure 15 and 16).
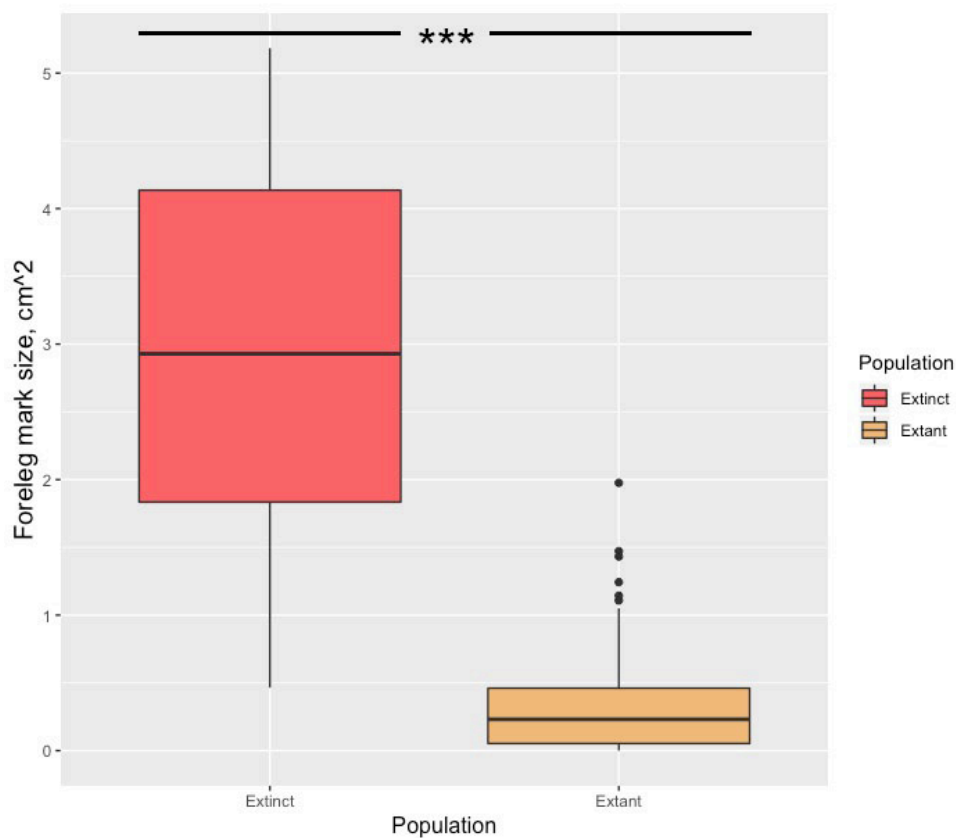
Figure 14. Boxplot of foreleg mark size in the extinct (n = 12) and extant (n = 72) population. The extinct population carried larger melanin marks than the extant population (W = 31, p < 0.001, Mann-Whitney U-test)
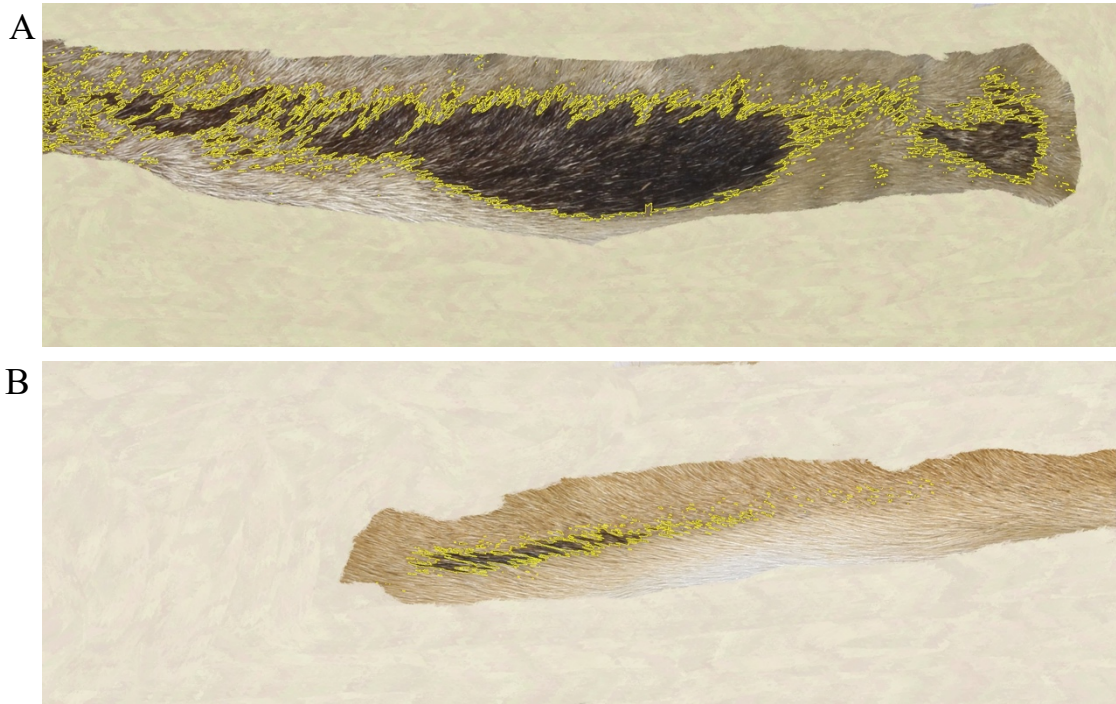
Figure 15. Processed images of foreleg marks from wolf hides, displaying the 300 cm^2 region of interest. Outlined area indicates the area that was identified as melanized. **A**: foreleg mark of a representative wolf hide from the extinct population (Museums no. 2650, NHM Oslo). **B**: foreleg mark of a representative wolf hide from the extant population (Museums no.22099, NHM Oslo)..

Figure 166. Hides photographed at the Natural History Museum in Oslo, **A**: Foreleg of a wolf from the extinct population (Museum no. 2649, NHM Oslo). **B**: Foreleg of a wolf from the extant population (Museum no. 11204, NHM Oslo).

4 Discussion

4.1 Methodical considerations

A main aim of this study was to develop a method for obtaining standardized methods for obtaining photographic documentation and morphological measurements to characterize phenotypic traits in wolves. Before discussing the results of the study, I will recapitulate some methodological considerations.

Firstly, obtaining reliable data from intact specimens in the field incurred a range of challenges. The time specimens were available in the field varied from some hours to two days, however most animals were only available for sampling a few hours between the animal's death and the planned skinning of the hide. Most often, hunters or contracted taxidermists kindly postponed their work, sometimes to late evening or night, for us to reach the site and perform the registrations. It must thus be considered a methodological weakness that morphological measures could not be taken twice, to obtain an indication of repeatability. When time again becomes available, I will however assess what measures can be checked against the standard background used for both skins and intact animals.

Furthermore, equipment needed to be available at short notice and with rigging time as efficient as possible. In Scandinavia, the hunt for wolves are usually carried out in the winter months and daylight is consequentially limited. Applying extra light sources was required in most of the photoshoots, as specimens usually became available for photographing and measuring in the afternoon. To avoid creating hard shadows, two strong high-lumen lamps were placed above head height. Ideally, applying more lamps would make rigging to avoid shadows easier. However, this would mean a heavier load of equipment to bring in the field and rigging time would be longer. In addition, some locations were lacking power outlets and the car battery was then used as a power source, limiting available power. Arrangements were however sufficient for lighting conditions to remain constant throughout each photoshoot, thus shooting in natural light when light conditions are changing fast is not advisable. However, if shooting under stable light conditions is not an option, camera settings should be recalibrated when necessary and the color checker passport visible in every image. Taking photographs before taking measurements proved to be important, as some specimens were

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still bleeding to some extent and minimizing bloodstains was critical for the following image analysis.

From museum collections, skull morphometrics were obtained from the extinct and extant Scandinavian population, and a total of 16 measurements were taken. Repeatability of these measurements was testable, and was carried out for three variables resulting in > 98% correlation between repeats indicated by linear regression analysis. While also suffering from highly unequal sample sizes in the museum material, the developed method for photo capturing and image analysis appeared robust, with high repeatability between observers, and capable of detecting between-population variation.

4.2 The natural history of the grey wolf in Scandinavia

Notably, interpretation of the current results requires some knowledge of the natural history of the wolf populations in concern. Wolves were once widely distributed throughout the Northern hemisphere occupying a wide range of habitats (Mech & Boitani, 2003). Early human cultures with a hunter and warrior way of life admired wolves for their hunting techniques, and some cultures strived to emulate the wolf´s way of hunting (Boitani, 1995). Their view of the animal was often reflected in the religious beliefs of these cultures, were the wolf was given godlike status (Boitani, 1995). The shift in attitude towards the wolf changed when human cultures started keeping and protecting livestock (Fritts et al., 2003). As human settlement spread, and the cultivating of land expanded, wolves and humans increasingly came in conflict (Boitani, 1995; Fritts et al., 2003). This was the beginning of a radical human persecution of wolves in Europe which became well organized in the Middle Ages (Boitani, 1995). Consequently, wolf populations decreased and became fragmented, some became isolated and other became extinct (Ellegren et al., 1996). The Scandinavian wolf history followed the same pattern as the rest of Europe. Hunting statistics from 1846 to 1977 shows that hundreds of wolves were killed each year until wolf populations rapidly declined in the mid nineteenth century (Bjerve, 1978; Vila et al., 2003). The grey wolf in Scandinavia became legally protected in 1966 and 1973 in Sweden and Norway respectably, but the population was then considered functionally extinct (Wabakken et al., 2001).

In 1983, wolves were again reproducing in Scandinavia, as a breeding pair of wolves was discovered in Värmland (Wabakken et al., 2001). They reproduced the following years, and a

new wolf population was established on the Scandinavian peninsula. The population consisted of under 10 individuals during the 1980s and genetic variability was lost due to inbreeding (Ellegren et al., 1996). In 1991, a new male contributes to the population, and the population started to grow exponentially (Vila et al., 2003). These three individuals still contribute with over 95% of the variation in the present gene pool (Jansson et al., 2015). Around the same time as wolves reappeared in Scandinavia, ungulate populations had undergone a rapid increase in population size (Austrheim et al., 2008), resulting in an abundance of prey for the new population (Olsson et al., 1997).

Today, the Scandinavian population is estimated to consist of around 400 individuals (Wabakken et al., 2018). The geographical origin of the source population is assumed to be the neighboring populations in Finland and/or Russia, with around 750 km to the closest breeding packs (Seddon et al., 2006). The dispersal corridor between the two populations is narrow, and few individuals make it to the Scandinavian population, and even fewer reproduce (Vila et al., 2003; Seddon et al., 2006). In view of the above, it should be noted that the morphological differences documented between the extinct and extant population must be interpreted and discussed while keeping in mind that they could represent random founder effects, geographical origin, direct environmental influences or even rapid adaption to the current environment.

4.3 Results in brief

As mentioned, regarding craniometric measures from museum specimens, only adult males were considered, with a final number of 11 representatives of the extinct population and 47 from the extant population. The results from the PCA showed that the first component explained 61.7% of the total variance found, and the three following components explained just over five percent each. The six variables that contributed most to PC1 are all various measurements of skull length and the most contributing variable was Condylobasal length (CbL). In PC2 the most contributing variables were measurements of skull width, for PC3 the most contributing variable was forehead slope, and measurements representing nasal length contributed most to PC4. The principal component analysis indicated a separation of the two populations on PC1 and PC3, but not on PC2 and PC4. The individual factor scores from the PC was indeed found to be significant between the populations on PC1 and PC3. A separate Mann-Whitney U-test was also conducted on the variables that contributed most to PC1 and

PC3 between the populations. Condylobasal length was significantly longer in the extant population than in the extinct population, indicating that the extant population has longer skulls than the extant. Forehead slope was significantly greater in the extinct population than the extant population, indicating a greater curve of the forehead in the extinct population. Correlation between age and these two variables (CbL and Fs) was tested to investigate if adult age had an impact on these skull measurements in each population. Variation in age was not related to CbL, and could thus not explain the difference between the populations. Age and forehead slope did not correlate in the extinct population, however, a weak but significant relationship between age and forehead slope was detected in the extant population. Linear regression between age and the four principal components was also tested, and no correlation was indicated. Notwithstanding the considerable difference in sample size between the populations, the possibility that age of the specimens contributed to Fs and hence PC3 variation should be considered as a possible explanation for this particular contrast between extinct and extant wolves. Length of the skull, on the other hand, must be assumed to diverge due to other biological factors (see discussion below).

Regarding foreleg melanin markings, the extinct population displayed distinctly larger marks than the extant population. Notably, the size of these melanin patches was not related to foreleg leg or length of the animal, indicating a functional difference in melanin synthesis underlying pronounced vs weak melanisation. Possible mechanisms will be discussed below.

4.4   Skull morphology

The grey wolf has adapted to a range of different habitats, and consequently different populations of wolves display different morphology in size, color and skull features (Milenvić et al., 2010). The variation in morphology between populations is greatest along the north-south directional axis (Nowak, 2003), and can be put in relation with the changes in environmental variables on the same axis (Pilot et al., 2006). My results indicate that adult male skull morphology differs between the extinct and extant Scandinavian wolf population. The differences found was related to skull length and the depth of the skull curve between the forehead and the nasal bone, and in short, the extant population exhibits a longer and flatter skull than the extinct population.

Pinpointing the causal factors behind this observation cannot be achieved within the scope of this thesis. I will however briefly discuss possible explanatory factors below. Environmental factors is one such possible driver for variation skull morphology. Local adaptions to different habitats could be the source to the variation in skull traits, and provided that the founding individuals were representative of their population, the extant population may share these traits with the source population. Intraspecific variation in morphology has been addressed by a number of studies, with an emphasis on skull variation. Such variation can occur over surprisingly narrow geographic and environmental ranges. For instance, Okarma and Buchalczyk (1993) found a difference in cranimetric caracters between mountain and lowland populations of wolves in Poland, where males from the mountain popuation had generally larger skulls than the males from the lowland population. Milenković et. al. (2010) studied morphometric variation in skulls between wolf populations in Serbia. They found that Carpathian wolves were larger than Dinaric-Balkan wolves and the latter had a more elevated snout and sagittal crest (Milenvić et al., 2010). Further, they present Bergmann´s rule (that larger size is an adaption to colder environments) as a possible explanation for cranial size differences. However, Bergmann´s rule is unlikely to explain the differences in skull traits found in the Scandinavian populations, as the source population of the extant population is likely to be found on comparable latitudes to todays core distribution area for wolves in Scandinavia. Notably, my results only suggest differences between the two populations in length of the skull and curve of the forehead, and not in overall skull size.

A vast range of environmental and/or genetic factors may have contributed to the observed divergence. Nutrient stress, for instance, affects skull growth patterns in mammals, particularly at the fetal stage (Pucciarelli et al., 1990; Gonzalez et al., 2014; O'Keefe et al., 2014). In Scandinavia, wolves mainly prey on ungulates, which also suffered a population decline concurrent with human expansion (Olsson et al., 1997; Austrheim et al., 2008). During the same time period as wolves were killed in large numbers, moose (*Alces alces*), red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) reached historically low population numbers, due to competition with domestic livestock and hunting pressure (Austrheim et al., 2008). Hence, the extinct wolf population in Scandinavia probably experienced a decrease in available resources due to a population decline in ungulates which reached critical low numbers around 1920. In contrast, the new established wolf population encountered an expeditious increase in ungulates, above all moose. Nutrient stress may thus have had an

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impact on the extinct population, as they probably suffered nutrient stress to some extent, however, no conclusions can be drawn from the available data.

The difference found between the extinct and extant population in skull length and curve of the forehead may reflect adaption to different hunting strategies or prey, either geographic or over time. Machado et al. (2018) investigated the stability and evolution of morphological integration of skull traits in Carnivora. They found a distinction between canids and other carnivores in changes in facial traits, especially snout length. Canids showed a higher flexibility and evolvability in facial trait involving snout and nasal length and the relative length of the face displayed the greatest variance (Machado et al., 2018). Such flexibility and evolvability in facial traits may have increased the canids capacity to respond to natural selection (Machado et al., 2018). Canids mostly rely on their head and teeth for apprehending prey, and the morphology of the jaw vary amongst canids based on diet (Slater et al., 2009) and hunting strategies (Figueirido et al., 2011). In carnivores, bite force, which correlates with gape angle, is a good indicator of feeding ecology (Figueirido et al., 2011). The length and breadth of the snout in canids reflect biteforce and loading stress in the skull when biting (Slater et al., 2009). Specifically, an elongated and narrow jaw is faster to close at the expense of bite force, while a short and broad jaw allows animals to produce a larger bite force while experiencing less stress on the skull (Slater et al., 2009). Wolves are hypercarnivore cursorial animals that usually hunt in packs (Van Valkenburgh, 2007). In contrast, solitary felids that take down large prey require a wide gape to constrain and suffocate prey, pack hunting hypercarnivores are less dependent on delivering a single bite with large bite force as their hunting behavior involves a joint effort of delivering repeated slashing bites to take down prey (Christiansen & Wroe, 2007; Figueirido et al., 2011). Even so, bite force is also important for pack hunting canids (Slater et al., 2009), and on the expense of a wide gape angle they have increased their bite force by lengthening the moment arms for the temporalis and masseter muscles in comparison to other large carnivores (Figueirido et al., 2011).

With this information in mind, the new established wolf population appeared in a habitat abundant with moose and little intraspecific competition. If their skulls were under selection pressure due to a shift in prey (from domestic livestock to moose), one might have expected to see longer skulls in the extinct population and a shortening of the snout in the extant population. However, this is not the case, and alternative explanations remain uncertain. A theoretical possibility is that wolves may not benefit from a shorter and broader skull, as they

already possess a high bite force compared to other hypercarnivore canids (Damasceno et al., 2013), Furthermore, a study conducted by Olsson et al. (1997) investigated the predation pattern of the wolf population in Scandinavia from 1988-1992, when the population size was around ten individuals or less. They found that wolves killed roe deer twice as often as moose, despite that the moose density was around three times larger than of roe deer. The possibility should also be considered that, despite the observed capacity for rapid evolution in canids, the observed pattern in skull morphology does not reflect adaptation, but random founder effects. After all, the contemporary population is considered to be solely the offspring of immigrants from an undefined region in neighboring countries. In summary, further studies should consider both founder effects, genetic drift, and inbreeding as possible mechanisms for the observed variation in skull morphology.

4.5   Foreleg melanin patches

When various wolf populations have been described in literature, a dark mark or stripe running down the front leg has been mentioned as a specific character (Pocock, 1935; Ognev, 1962; Pulliainen, 1965; Castelló, 2018). Here, I investigated the characteristic foreleg mark in the extinct and extant wolf population in Scandinavia and found that the mark differed significantly in size between the two populations. The extinct population had overall larger markings on their legs, while the extant population usually had a small stripe or no markings at all. Conceivably, a broader sampling range could have revealed an interesting interaction between season and life stage, since a difference between juveniles and adults were only present in the dataset obtained exclusively during the winter season (i.e. present day field data).

The observed color variation incur a wide range of possible functional explanations. Firstly, stripes or patches of black can serve as camouflage, and foreleg markings of some mammalian species have been suggested to be disruptive coloration (Caro, 2009). Disruptive coloration are sets of color marks that breaks up the outline appearance of an animal to prevent detection of the animals shape and are often in a contrasting color to the rest of the pelage (Caro, 2005; Stevens & Merilaita, 2011). Also, prominent black marks are found in numerous species of diurnal artiodactyla living in open landscapes and are thought to act as disruptive markings to break up the outline of the legs (Stoner et al., 2003; Caro, 2005, 2009). However, this theory in artiodactyla is disputed (Stoner et al., 2003), and there is little

evidence of disruptive colorization in predators. Breaking up the outline on the front of the body could indeed potentially disturb danger assessment by prey, say with regards to adjudging distance and direction of travel of approaching predators. A function in disruptive coloration is possible to reconcile with the observation that the extinct and extant populations display different sizes of the foreleg mark, given that the original population may have evolved in a different environment than current wolves (see discussion about geographical origin below).

Caro (2009) suggested that marks on the frontal surface of the forelegs of mammals could serve as communication signal, since many of these species live in large social groups. He further emphasizes that little is known about the placement and function of such marks. Colorization used in intraspecific communication may signal reproductive condition, health and dominance (Caro, 2005, 2011). Melanin patches found on the front of animals can also be signals of dominance (Järvi & Bakken, 1984; Caro, 2011). In this context, it is intriguing that field data suggested that juveniles express more foreleg melanisation than adults. Wolves display a highly complex and hierarchical social behavior (Packard, 2003), but it would seem controversial to suggest that juveniles generally enjoyed higher social rank than adults.

Like with skull morphology, nutritional and environmental factors may influence the development of melanin patches. A classic example is the male lion (*Panthera leo*) where dark mane is associated with high testosterone, longer reproductive span, higher nutrition and age (West & Packer, 2002; Caro, 2005). In particular, dark color of lions mane have been associated with high nutrition intake, where darker maned males have higher food intake than lighter maned males (West & Packer, 2002). It is not unlikely that the extinct and the extant population had different accessibility to food due to the low density of ungulates in a longer time period before 1960 followed by a rapid increase of moose around 1970s. The direction of this effect should however be exactly the opposite of what is observed, namely a weaker signal in the current population. If there is a linkage between melanin production in the foreleg of wolves and high nutrition, one would expect more coloration among the extant Scandinavian wolf population than the extinct, however, this is not the case in my results. However, the evidence support of linkage between nutrition and melanin production in lions have been questioned and the relationship between melanin production and high nutrition in mammals are less clear (Hill & McGraw, 2003).

Melanin is also associated with stress, in that animals that are less resistant to stress and disease show reduced melanin production compared to those that cope well with the current environment (Ducrest et al., 2008; Khan et al., 2016). Conceivably, migrants could be under stress during exposure to new environments, but it is hard to see how such an environmental effect could explain why we still see less pronounced melanin patches in the present populations (c.f. current field data). Contrasting geographical origin of the extinct and extant wolf population is perhaps a more likely explanatory factor for the observed color variation. An investigation of the distribution of foreleg mark in wolves in Finland, Russia and Scandinavia (extinct population) was done by Pulliainen (1965). He divided individuals in two groups, those with black mark and those without and found that both color morphs are found in Finland and Russia but not in Scandinavia, where all the individuals had distinct foreleg marks. Further, the most common morph was possessing a black mark on the foreleg, while individuals without the mark were more abundant in eastern parts of Finland (Pulliainen, 1965).

Pulliainen (1965) suggested that the reason for the different phenotypes in Finland could be related to the concept of subspecies of wolves, i.e. mixed breeding between the so designated tundra wolf (*Canis lupus albus*) and the Eurasian wolf *(Canis lupus lupus)*. The term subspecies is often used in the description of wolves from different geographical regions. Some studies designate about 12 subspecies of grey wolf (Sillero-Zubiri et al., 2004), and the color morphs vary between the different subspecies (Castelló, 2018). However, the term subspecies may be problematic for long-dispersing species such as wolves, as subspecies may interbreed and hybridize (Robert et al., 2003). The tundra wolfs coat color is described as light grey with some ochre shade and white paws (Castelló, 2018). It is not described with a black mark on the front of the foreleg to my knowledge. Their distribution range is the tundra zone of northern Europe and Asia (Nowak, 2003; Castelló, 2018). North-east of Finland seem to be a meeting point between the Eurasian subspecies distribution and the tundra wolf distribution (Nowak, 2003; Castelló, 2018), but how much the distribution of the subspecies overlap or if any overlap occur is not known for certain.

Potentially, the mark on the front leg of wolves may have adaptive value for animals living in woodland or forest and serve as a type of camouflage, whereas the absence of such stripe may be an adaption to tundra and an arctic environment. If interbreeding between two potential subspecies did occur, and heritability follows a non-Mendelian pattern, it could yield a

phenotype intermediate between a non-marked and highly marked variant. Such individuals could potentially be the founders of the new established wolf population in Scandinavia, given that Finland or Russia is the suggested origin of the population. Of note, interbreeding between subspecies is just a hypothesis and should be addressed in further studies. The foreleg stripe size found in the extant Scandinavian population may also be accurately representative of a still present source population, without any occurrence of interbreeding between subspecies or populations being involved.

5   Conclusions

This study documents morphological differences between two wolf populations inhabiting the same geographical area in two different time periods. Data was obtained on craniometric measures and size of the melanin patch found on forelegs. Significant differences between the extinct and extant Scandinavian wolf populations were observed in measures related to skull length and forehead slope, with the current population exhibiting a longer skull with less pronounced curvature between the forehead and nasal bone than the extinct population. Possible explanations for different skull morphology in these two wolf populations could be adaptation to different environments, differences in attainable nutrition, or genetic founder effects followed by inbreeding and/or random genetic drift. Foreleg melanin patches were significantly larger in the extinct compared to the extant population. In other species distinct melanin patches indicate good condition, social dominance, and stress and disease resistance. Alternatively, in wolves this trait may bring adaptive value to individuals inhabiting forests by serving as disruptive coloration in this type of landscape, since wolves inhabiting tundra and arctic open landscapes are reported to not exhibit this trait. The small foreleg mark size observed in the extant population may be a trait shared with the source population, and the possible function of this trait in wolves should be further investigated. Further studies are also needed to address proximate and as well as ultimate causes of the observed differences in skull morphology between the study populations.

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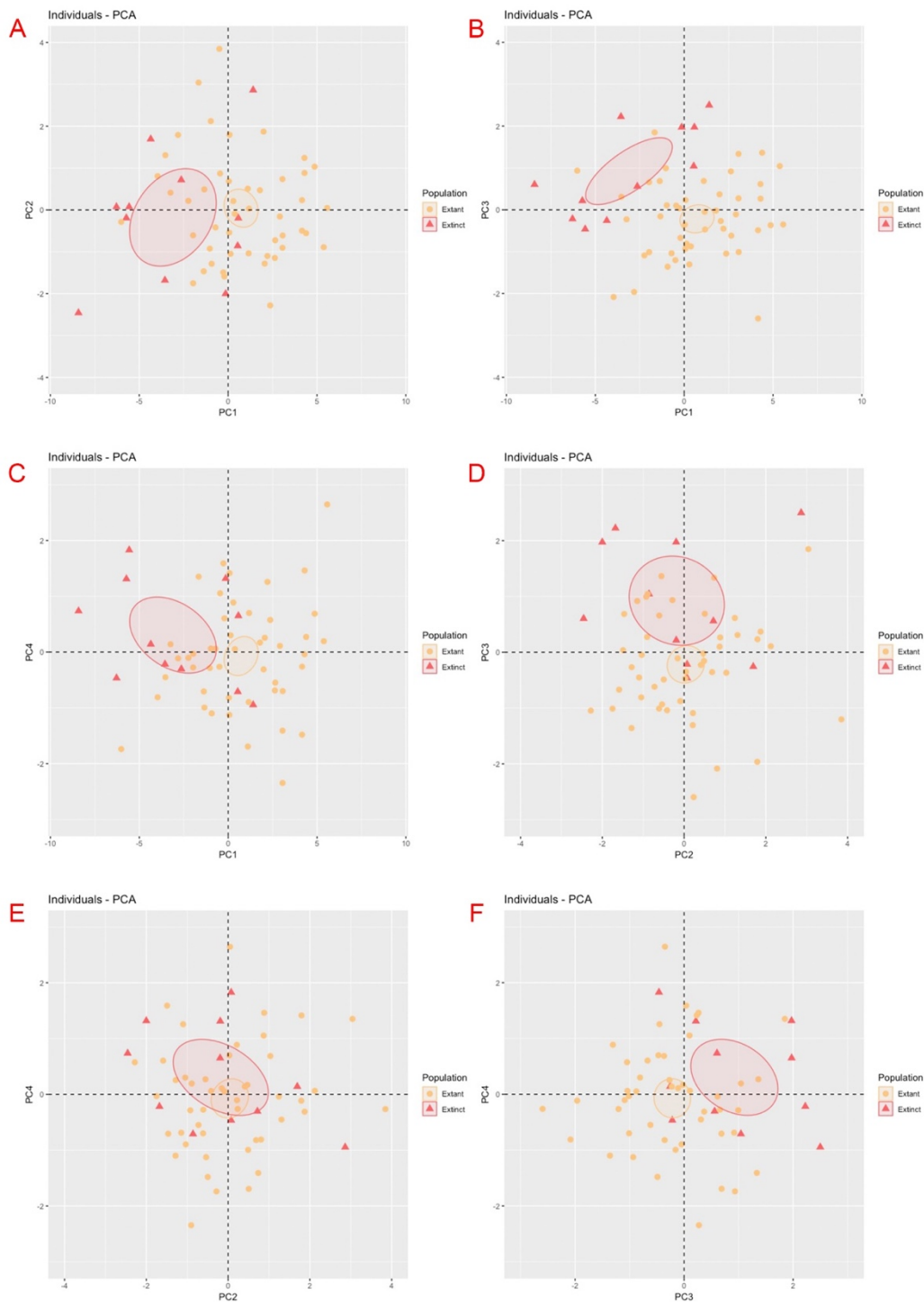
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Appendix

A 1. Variable factor map that shows correlation between the variables and the components. **A:** PC1 vs PC2, **B:** PC1 vs PC3, **C:** PC1 vs PC4, **D:** PC2 vs PC3, **E:** PC2 vs PC4, **F:** PC3 vs PC4

A 2. Individuals factor map that shows individuals placement in the principal component analysis. Ellipses illustrates groups mean placements. Extinct individuals are displayed with red triangles. Extant individuals are displayed with yellow circles

 A: PC1 vs PC2, **B:** PC1 vs PC3, **C:** PC1 vs PC4, **D:** PC2 vs PC3, **E:** PC2 vs PC4, **F:** PC3 vs PC4