Faecal sampling as a non-invasive population monitoring and management method for reindeer and caribou

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Abstract

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Methods to monitor and manage populations of reindeer (*Rangifer tarandus*) and caribou are often impractical, costly, and can be harmful to the animals. Non-invasive techniques that produce high quality and accurate results are better alternatives. We collected blood and faecal samples during 2008 - 2010 from two herds of Fenno-Scandian reindeer to explore the potential of using faecal samples to determine the age class (calf, yearling and adult), the sex, as well as the reproductive status of individuals. This was done using two main techniques: faecal progesterone metabolite concentrations (specifically faecal pregnane) and faecal pellet morphometrics. Faecal pregnane was highly correlated with plasma progesterone and therefore, in theory could be a substitute for traditional hormone procedures. We were not successful in using faecal pregnane for sexing or aging individual reindeer. Using plasma progesterone and faecal pregnane, we were able to determine a pregnancy cut-off value for all of the study reindeer, and were therefore able to clearly differentiate pregnant from non-pregnant females. Using a combination of pellet dimensions including maximum length, width, and depth, we were able to differentiate with high accuracy, three age classes of reindeer (calf, yearling, and adult). A combination of faecal sampling using pregnane and pellet size, with the addition of current faecal DNA techniques, may allow the collection of important

population data essential for monitoring and management of wild and elusive species such as wild reindeer and woodland caribou.

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Contribution of authors

As the first author, I was responsible for the conception, design, set-up, some data collection (Finland), part of the laboratory work, and the data analysis. The manuscripts and thesis were also written by myself.

Chapter 1 was co-authored by Dr. Robert B. Weladji, Dr. Erik Ropstad, Ellen Dahl, Dr. Øystein Holand, Dr. Gabriela Mastromonaco, and Dr. Mauri Nieminen who all served as mentors and supervised the work. Dr. Erik Ropstad helped with the Svalbard data, in addition to advising on the laboratory analysis with Ellen Dahl. Dr. Gabriela Mastromonaco assisted with the lab analysis and corrected an earlier version of the manuscript. Dr. Øystein Holand advised with the experimental design and the coordination of the data collection process overseas, and edited an earlier version of the manuscripts. Dr. Mauri Nieminen further helped with data collection in Finland.

Chapter 2 was co-authored by Dr. Robert B. Weladji, Dr. Erik Ropstad, Ellen Dahl, and Dr. Øystein Holand, all who mentored the project. Dr. Erik Ropstad helped with the Svalbard data collection and allowed access to the samples and laboratory as well as correcting an earlier version of the manuscript. Ellen Dahl provided assistance in the lab. Dr. Øystein Holand again assisted with the coordination of the data collection in Finland and corrected an earlier version of the manuscript.

Dr. Weladji further assisted with the statistics and correction of this thesis.

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General Introduction

Knowledge of demographic parameters is essential for monitoring populations of wild and domesticated animals (Mysterud and Ostbye 2006). Techniques to gather population information such as population size, sex and age structures are generally impractical and costly, especially for species that are elusive and rare; therefore it is of interest to seek better, more economical methods that can produce similar high quality, accurate results (Kohn et al. 1999, Creel et al. 2003). Some of the traditional tools used to monitor animal populations can be quite physically and behaviourally harmful to the animals as they generally require some sort of direct contact, whether in the form of capture by darting or netting or by visual observations from a helicopter. Both methods have been shown to negatively affect the survival of the targeted individual and consequently, many species' reproductive success (see e.g. Larsen and Gauthier (1989) for moose (*Alces alces*), Spraker (1993) for general wildlife, Côté et al. (1998) for mountain goats (*Oreamnos americanus*), DelGiudice et al. (2005) for white-tailed deer (*Odocoileus virginianus*)). Because of its benefits, non-invasive approaches appeal to many scientific fields including behavioural ecology, reproductive biology, and conservation (Goymann 2005).

This study examines the use of non-invasive monitoring techniques for the purpose of conservation and management of wild reindeer and especially woodland caribou (*Rangifer tarandus caribou*) in Northern Canada. Populations of this species have been in decline throughout the last century due to habitat loss and increased predator access (Edmonds 1991, COSEWIC 2002, McLoughlin et al. 2003). Status reports from 2002 listed woodland caribou populations as being anywhere from extinct (Dawson's

herd from Queen Charlotte Island) to not at risk (Newfoundland's population), although the majority of the populations are considered threatened. Because some of these populations are small in size, they are of great conservation concern because of their vulnerability and threat of extinction (Soule 1987). A comprehensive action plan is required for the long term management and conservation of woodland caribou. In order to be reliable, such action plans should be based on trustworthy biological data including population structure (sex and age structure even at a coarse scale) and recruitment rate. Unfortunately, due to the vast habitat range of this species, the multitude of sub-populations, as well as time and financial constraints, successful monitoring of woodland caribou has been neglected (Edmonds 1991). Therefore, in an effort to minimize invasiveness, an easier, less costly and time efficient method is required for effective management of all Canadian populations.

With non-invasive survey methods, animals are generally not subjected to stress or risk of injury; yet the studies being carried out can be rigorous. For species that can be difficult to locate such as the woodland caribou, and where stress may negatively influence body condition threatening their survival, the collection of faeces may be a superior alternative to the regular capture-mark-recapture methods (Kohn and Wayne 1997, Kalinowski et al. 2006). Faecal samples, in particular, can be easily obtained without having to disturb the animals, especially over the winter months when they can be found on the snow. Sampling can also be done continuously and regularly as access to the faeces is the only major factor for collection, so it is a superior method for research on wildlife and stress-prone species (Millspaugh and Washburn 2004, Schwarzenberger 2007). Faecal analysis holds the potential to provide information about genetics, life

history, and population dynamics of mammals (Kohn and Wayne 1997). In particular, much work is currently being done on the use of DNA found in faeces to identify individuals for capture-mark-recapture studies allowing for the estimation of population sizes of specific species (see e.g. Kohn et al. (1999) for coyote (*Canis latrans*), Creel et al. (2003) for wolf (*Canis luprus*), Wilson et al. (2003) for European badger (*Meles meles*), and Hettinga (2010) for woodland caribou). Some other applications of faecal molecular techniques could be the identification of a species' sex, home range, reproductive patterns, and kinship (Kohn and Wayne 1997). Although the use of DNA has many strengths, population parameters such as pregnancy diagnosis and age classification cannot be estimated using this method alone.

Another possible technique to identify population parameters such as reproductive status of individuals, sex, and age may be the use of faecal steroid concentrations levels. Faecal hormone quantification is another non-invasive technique which may be used alone or in conjunction with faecal DNA or other methods including pellet morphometry. Previous studies have focused mainly on the use of stress and sex hormones found in faecal samples. Patterns in the concentration levels of faecal hormones have been successfully used to assess female reproductive status (using mainly progesterone and oestrogen) (see e.g. Monfort et al. (1993) for moose, White et al. (1995) or elk (*Cervus elaphus nelsoni*), Wasser et al. (1996) for African elephants (*Loxodonta africana*), Schwarzenberger et al. (1999) and Kusuda et al. (2007) for Okapi (*Okapia johnsoni*) and Vors et al. (2007) for woodland caribou). Messier et al. (1990) also found a difference in faecal progestin levels of caribou between non-pregnant fawns and adult females, but White et al. (1995) did not report any age effect between male, calf or non-pregnant

female elk. Faecal hormones, as a possible tool to monitor populations, has not yet been extensively studied; however it has been noted that the possibilities are great (Ball et al. 2007).

Another tool that may be helpful for non-invasive caribou age estimation is the use of faecal pellet size to identify individual age classes. Many studies have attempted to use pellet size or dimensions to predict age (MacCracken and Van Ballenberghe 1987, Reilly 2002, Chapman 2004, Southgate 2005, Ball et al. 2010). Southgate (2005) was able to differentiate between immature individuals and adults using the relationship between animal weight and faecal pellet size of the greater bilby (*Macrotis lagotis*). Uye and Kaname (1994) examined the correlation between body length/body carbon weight and faecal pellet size of zooplankton. Their study discovered a strong correlation between body size and faecal pellet size, showing that larger zooplankton will excrete larger faecal pellets. Since *R. tarandus* body size increases as the individual ages to adulthood, there is potential that pellet size may indicate individual age and hence the age structure of a population. More recently, Ball et al. (2010) found a relationship between pellet size and caribou age however, this remains to be investigated for wild reindeer.

Amongst the information required for proper population monitoring, knowledge of the age structure is essential in exploring trends in recruitment, population growth, mortality, and reproductive status of the population (Reilly 2002). This study involves the examination of the use of both faecal hormones and faecal pellet size excreted by *R. tarandus* to acquire important information about their population. I had four main objectives: (1) to determine if faecal pregnane reflects the circulating progesterone levels in the animal; (2) to explore the possibility of identifying female reproductive status

(pregnant or not pregnant) using reindeer fecal pregnane; (3) to investigate whether the sex and the age class (calf, yearling and adult) of individual reindeer can be assessed using faecal pregnane; and (4) to explore whether age class can be determined by faecal pellet size of a reindeer.

Chapter 1:

Faecal hormones as a non-invasive population monitoring method for reindeer and caribou

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Abstract

Proper management of threatened species requires prior knowledge of population sizes and structures, however current techniques to gather this information are generally impractical, costly, and can be stressful on the animals. Non-invasive methods that can produce high quality and accurate results are better alternatives. In winter 2010, blood and faecal samples were collected from two reindeer (*Rangifer tarandus*) populations (Kaamanen, Finland and Svalbard, Norway), to investigate the feasibility of using faecal progesterone metabolites to help estimate the reproductive status, the sex, and the age structures of the populations. We first examined the relationship between plasma progesterone and faecal pregnane concentrations. We further assessed whether faecal pregnane levels would clearly differ between calf, yearling, and adult; and between pregnant and non-pregnant females. Using standard procedures, we quantified faecal pregnane and plasma progesterone of females and males of different ages from the two herds. We found in both populations, a significant positive relationship between faecal and plasma progesterone levels. However, the range of faecal pregnane concentration was much wider in Finland than in Svalbard, possibly due to differences in diet or body condition. We determined a cut-off value of 2.34 ng/ml plasma progesterone and 2469.4 ng/g dried faecal pregnane to identify pregnant reindeer from non-pregnant animals. We found a significant difference in faecal pregnane concentrations only between calves and yearlings/adults in Finland. We could not differentiate between males, non-pregnant adults, or calves of either sex; therefore identification of sex may have to rely on the use of DNA techniques. Our results suggest that hormone concentration, in combination with faecal DNA and pellet morphometry techniques, may provide important population

parameters and is a valuable tool for the monitoring of reindeer as well as threatened populations of woodland caribou throughout the winter and early spring.

Introduction

Management and conservation of wild animals requires knowledge of demographic and population parameters. Ungulate population dynamics depend on the sex ratio, age structure, and reproductive status, amongst other measurements, therefore accurate estimates of these features are essential for future management and conservation (Mysterud and Ostbye 2006). Age structure varies within populations over time and knowledge of these differences is critical for exploring trends in recruitment, population growth, survival, senescence, and reproductive status (Reilly 2002, Festa-Bianchet et al. 2003). Age-specific reproduction alone could aid our understanding of population performance and dynamics (DelGiudice et al. 2007) and could help predict future trends in population size (Bergerud 1992). Often, the techniques involved in gathering such information are distressing to the study animal (Côté et al. 1998, Cattet et al. 2008, Omsjoe et al. 2009) and can be extremely costly (Haigh 1979, Valkenburg et al. 1983). Much of the current ungulate research involves a combination of the following methods: field observations by helicopter, physical control (capture via darting or net gunning), chemical immobilization, measurements of physical attributes, ear tagging, radiocollaring, and sample collections from the animal including blood, teeth, hair, and faeces (DelGiudice et al. 2005).

Blood is the traditional medium for examining hormones that can provide information on female reproductive status [via progesterone (P4) or estradiol; (Ropstad et al. 2005, Shipka et al. 2007)], stress levels [via cortisol; (Denicola and Swihart 1997, Li et al. 2007)], and mating and aggressive behaviour in males [via testosterone; (Whitehead and McEwan 1973, Li et al. 2004)]. Physical manipulation of animals has been shown to

directly modify the concentrations of these sex hormones by increasing stress levels (Wesson et al. 1979, Barja et al. 2008, Togashi et al. 2009). Unlike blood collection, faecal sampling does not require special training of personnel, can be gathered with ease in the field, and does not require direct contact with the animal (Goymann 2005).

Pregnancy can be determined by ultrasound or by measuring pregnancy-related biochemical markers (e.g. reindeer; Ropstad et al. 1999). Progesterone, specifically in blood and urine, has been commonly used for pregnancy diagnosis in many species, including reindeer and caribou (Lasley and Kirkpatrick 1991). After conception, P4 increases in the blood quite steadily until parturition when levels drop rapidly (Ropstad et al. 2005). Faecal P4 has been used in several species to discriminate pregnant and non-pregnant females (Schwartz et al. 1995, Wasser et al. 1995, Schwarzenberger et al. 1996, Brown et al. 1997, Kusuda et al. 2007). Given that the gestation period of reindeer and caribou spans from October to June (COSEWIC 2002) and ovarian cycling stops over winter, detecting their reproductive status by collecting faecal samples from the snow would be, in theory, a feasible way to determine the pregnancy rate of a population.

Success of using sex hormones to help determine the sex or age of an animal is still quite rare. Sex determination in wild populations is important to understand the dynamics and structure of populations, differential habitat use, breeding behaviours, and how to effectively manage the species (Barja et al. 2008). Sex typing through sex hormones such as estradiol, progesterone, and testosterone (T) has been successful in birds (Cockrem and Rounce 1994) and several species of wolves (Velloso et al. 1998, Soto et al. 2004). Recent studies have used androgens for age determination. Castro and Sousa (2005) showed that faecal androgen levels vary significantly between adults and

juveniles in common marmoset (*Callithrix jacchus*). A study by Rooney et al. (2004) found that hatchling American alligators (*Alligator mississippiensis*) had a range of T significantly lower than adults (Rooney et al. 2004). Lynch et al. (2002) also found that the mean subadult T level was significantly lower than the mean adult T level throughout the year in wild male tufted capuchin monkeys (*Cebus apella*). The use of faecal P4, on the other hand, has been unsuccessful in identifying age class in female spider monkeys (*Ateles geoffroyi*) (Hernandez-Lopez et al. 2010). Progesterone is secreted by the corpus luteum and placenta during pregnancy, therefore a certain amount of P4 is required by all animals to maintain pregnancy (Flood et al. 2005, Ropstad et al. 2005). Circulating levels of estradiol and P4 (females) or T (males) have been found in other organisms to decline with age as a result of senescence (Chanson et al. 2004), suggesting that the use of P4 to estimate not only reproductive status, but also age class may be possible in reindeer and caribou.

In this paper, we explored the possibility of using faecal sampling as a possible monitoring technique for wild and captive reindeer with the hope that the results may be transferrable to woodland caribou (*R. t. caribou*). It is essential to perform studies on both captive and wild populations to account for possible external effects such as differences in diet, stress levels, body conditions, and environmental conditions. Populations of woodland caribou have been decreasing during the last decade, mainly due to habitat loss and predation (McLoughlin et al. 2003). Most populations are considered to be threatened by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC 2002). Unfortunately, due to their vast habitat range and the high cost of monitoring programs, the status of nearly 50% of the North American populations

are unknown (Edmonds 1991). It is in our best interest to search for techniques that are safe for the animals, cost efficient and still produce high quality, accurate results.

Progesterone held the most promise for this study as the faeces were collected during winter when other sex hormones such as testosterone and oestrogen are at base levels or are fluctuating (Bubenik et al. 1997, Shipka et al. 2007). We first validated the extraction and assay procedures for faecal pregnane in reindeer. We also investigated the relationship between faecal and blood hormone levels in reindeer in order to assess whether faecal hormone concentrations reflect the total hormone levels found in plasma. We further explored the possibility of identifying female reproductive status (pregnant or not pregnant), sex, and age class (calf, yearling and adult) of reindeer through faecal P4. Finally we tested whether faecal P4 levels differed between the semi-domesticated and wild reindeer populations.

Material and Methods

Study Area

This study used data from two reindeer populations, the Svalbard herd and the Finland herd. The first population was located in Nordenskiøldland, Spitsbergen (77°50` – 78°20` N, 15°00` – 17°30` E), Svalbard, Norway. There were approximately 1500 wild Svalbard reindeer (*R. t. platyrhynchus*) in this herd, although large annual population fluctuations are common due to the extreme environmental conditions present (Milner et al. 2003). The reindeer feed naturally on winter browse of mosses and low-quality vascular plants (Bjorkvoll et al. 2009). The herd has been followed since 1994 and female reindeer are caught and monitored each year for individual survival and fecundity.

A large proportion of the females have been marked with ear tags and neck collars for identification and are of known age (Milner et al. 2003).

The second reindeer population was located at the Kutuharju reindeer field station (69ºN, 27°E) near Kaamanen, Northern Finland. The herd consists of approximately 100 reindeer (*R. t. tarandus*) managed by the Finnish Reindeer Herders Association. Continuous collection of important information on the ecology and behaviour of reindeer has been ongoing since 1969. The semi-domestic reindeer are free-ranging within large enclosures (~ 45 km^2) most of the year, excluding the calving period when they are confined to a small calving area (~ 50 ha) and during the rut when they are confined to two fenced areas: Sinioivi (15 km^2) and Lauluvaara (13.8 km^2) (Eloranta and Nieminen 1986, Holand et al. 2003). Four to five times a year, the animals are briefly rounded up into smaller enclosures while they are sexed, weighed, and calves are marked with ear tags for identification. The animals are sometimes supplementary fed during winter as well as during these round-ups. Feed contains lichens, molasses, and pelleted concentrates (Eloranta and Nieminen 1986).

Data Collection

Svalbard reindeer

Methods of capture are described by Milner et al. (2003). Faecal and blood samples were collected from reindeer (n = 66) during February 2010. To ensure accuracy of the hormone measurements, the faeces were either gathered directly from the anus or from the snow immediately following excretion (Goymann 2005). The faecal and blood samples remained frozen at -20° C until analysis. The study was carried out in agreement with the provisions enforced by the Norwegian National Animal Research Authority. All

activities related to capturing and sampling animals are done with permission from the authorities of Svalbard. Of the 66 faecal groups collected from female Svalbard reindeer, 62 pellet groups were from adults, 3 pellet groups were from yearlings, and 1 pellet group was from a calf.

Finland reindeer

Faecal and blood samples (n = 76) were collected during two round-ups in January and March 2010. Blood was collected into heparinized tubes via jugular venipuncture followed by faecal sampling directly from the anus into plastic bags. The samples were collected in agreement with the Animal Ethics and Care certificate provided by Concordia University and in accordance with the regulations set by the Finnish National Advisory Board on Research Ethics. The blood samples were put on ice overnight and then centrifuged the following day for 15 minutes at 3,000 x *g*. Blood and faecal samples were stored at -20°C until analysis so as to slow microbial activity and reduce immunoreactivity problems (Millspaugh and Washburn 2004, Palme 2005, Capezzuto et al. 2008). We collected 40 blood and faecal samples in January 2010 [Adult (16♀, 6♂); Yearling (3♀, 5♂); Calf (5♀, 5♂)] and 36 blood and faecal samples in March 2010 [Adult (15♀, 4♂); Yearling (3♀, 5♂); Calf (5♀, 4♂)].

Plasma analysis

All frozen plasma and faecal samples were shipped to Oslo at the Hormone Laboratory of the Norwegian School of Veterinary Science, Department of Production Animal Clinical Sciences for further analysis. Plasma levels of total P4 were determined by a solid-phase radioimmunoassay kit (Spectria® Progesterone ^{125}I Coated Tube RIA, Orion Diagnostica, Espoo, Finland). The assay was previously validated for use with reindeer

plasma by demonstrating parallelism between dilutions of reindeer plasma samples and a standard curve and by recovery of unlabeled ligand (Ropstad et al. 2005). No modifications to the standard procedures were needed. The detection limit of the assay was 0.094 ng/ml. Intra-assay coefficient of variation (CV) was less than 5%. Inter-assay CVs were 8.8% (0.39 ng/ml), 7.6% (5.98 ng/ml), and 5.9% (13.85 ng/ml) (n = 18).

Faecal analysis

The faeces were dried at 60°C overnight before extraction to remove any water. Each sample was homogenized (for even distribution of the metabolites within the faeces) and was subsampled for analysis (Goymann 2005). Faecal sample extraction followed procedures described by Kummrow et al. (2010). In brief, approximately 0.2 g of mixed dry faeces was added to 5 ml of 80% methanol (in distilled water). The sample was vortexed briefly and left to rotate overnight at room temperature. The sample was centrifuged the following day for 15 minutes at 1,200 x *g* and the supernatant was decanted and stored at -20°C.

Efficiency of the extraction procedure was analyzed through recovery of exogenous hormone (P4) added to the faecal samples before extraction. Faecal samples from captive reindeer held at the Toronto Zoo were used. Faecal extraction efficiencies were determined from five faecal samples. The percent efficiency was calculated using the following formula: amount observed/amount expected x 100%; where amount observed is the value obtained from the spiked sample minus background and amount expected is the calculated amount added. The percent efficiency is presented as mean ± SE (88.50 ± 2.00%) and is considered sufficient for this study.

Faecal steroid metabolites were quantified using enzyme immunoassay (EIA) methods previously described by Kummrow et al. (2010). Pregnane antiserum and horseradish peroxidase conjugate (C. Munro, University of California, Davis, CA) were diluted to 1:8,800 and 1:40,000, respectively and progesterone standards were run at 15.6 – 4,000 pg/ml.

Pregnane assay validation

Parallel displacement between the standard curve and serial dilutions of faecal extract was used to detect immunological similarities between the standard and sample hormones. A representative pooled sample of faecal extracts was serially diluted two-fold from 1:2 to 1:512 in assay buffer and run alongside the standard curve. The graphs were plotted as relative dose versus percent antibody bound and linear regression analysis of the resulting curves was performed.

To assess repeatability of results, calculation of intra- and inter-assay coefficients of variation (CVs) were performed. Intra-assay CVs were consistently monitored on each plate in real time by examining the CV of each duplicate run on the plate. Only values from duplicates with < 10% CV were recorded as data (G. Mastromonaco personal communication). Intra-assay CVs were further evaluated using a pooled extract at 50% binding loaded repeatedly across three plates. Inter-assay CVs were evaluated using faecal extract controls (30 and 70% binding) loaded in duplicate on each plate.

To examine possible interference of components within the extract with antibody binding, recovery of a known amount of hormone was calculated for each hormone assay. A pooled sample of faecal extracts diluted to the usual range for unknown samples was used. To 100 µl of pooled extract, 100 µl of increasing concentrations of hormone

standard were added in the range used for the standard curve. For each assay, the diluted pool was assayed alone to determine endogenous hormone levels. The percent recovery was calculated using the following formula: amount observed/amount expected x 100%, where amount observed is the value obtained from the spiked sample and amount expected is the calculated amount of standard hormone added plus the amount of endogenous hormone in the unspiked sample. The graphs were plotted as hormone added versus hormone recovered, and regression analyses were used to determine if there was a significant relationship between them.

High Performance Liquid Chromatography

Characterization of steroid metabolites (number and relative proportions) in the faecal extracts was done using high performance liquid chromatography (HPLC), according to the method used by Kummrow et al. (2010). In brief, pooled faecal extracts were purified by solid-phase extraction using Sep-Pak C18 cartridges (Waters Scientific, Mississauga, ON, Canada) prior to HPLC analysis using a Nova-Pak C18 column (Waters Corp., Dublin, Ireland), connected with a Security Guard C18 guard column (Phenomenex, Torrance, CA). Twenty-one reference steroid hormone standards (Steraloids Inc., Newport, RI, USA) and the hormone sample were eluted at room temperature using a gradient of acetonitrile and water (27.5:72.5 – 15:85). Eluent fractions were collected every 30 sec. The fractions (300 µl) were dried down under the flow of nitrogen gas, re-suspended in equal volumes of EIA buffer, and assayed in duplicate to determine immunoreactivity.

Statistical Analysis

We used simple linear regression to examine the relationship between plasma P4 and faecal pregnane (PROC GLM using the SAS 9.2 software; SAS Institute Inc. 2008). We log-transformed both blood and faeces data for Finland and Svalbard to achieve normality and constant variance of the residuals.

Reproductive status was assessed in Finland by presence of a calf and in Svalbard by transrectal real-time ultrasonography performed in February 2010 (Scanner 400, 5 MHz, linear transducer, Pie Medical, the Netherlands). Since the collection period occurred late in the winter when reindeer had either stopped cycling due to pregnancy or no fertilization, we did not expect a great difference between the P4 levels between these two months. Indeed, we found no month effect on either blood or faeces after controlling for the effect of sex and reproductive status (Linear model using PROC GLM in SAS; $F_{1,74} = 0.865$, $p = 0.36$ and $F_{1,74} = 0.002$, $p = 0.97$ for blood and faeces respectively) and month was removed from subsequent models. Mixed models [PROC MIXED using the SAS 9.2 software; SAS Institute Inc. 2008] were also used to test whether P4 levels in blood plasma and pregnane in faeces (1) differed between pregnant and non-pregnant females, (2) differed between the Finland and Svalbard females while controlling for pregnancy status, using square-root plasma P4 and log faecal pregnane transformations to achieve normality and constant variance of the residuals. Mixed models [PROC MIXED in SAS] were used for the Finland herd and linear models [PROC GLM in SAS] were used for the Svalbard herd (3) to test whether P4 levels in plasma and pregnane in faeces differed between the three groups with low P4 levels (calves, non-pregnant females, and males) (using an inverse transformation for faecal pregnane) (the denominator degrees of

freedom (DDF) for this mixed model was determined by using the Kenward-Rogers approximation (Littell et al. 2006)) and (4) to determine age class (calves, yearling and adult) from the hormone level controlling for reproductive status and month (using log transformations for plasma and faecal data). Both herds and sexes were analyzed separately. "Individual ID" was included in our mixed models as a random factor to account for repeated measurement on the same individuals, thereby controlling for pseudoreplication. To determine the most accurate cut-off concentrations to diagnose pregnancy, we compared the P4 levels found in plasma and faeces of pregnant and non-pregnant females. All pair-wise comparisons were performed using Tukey adjustment. A significance level of $p < 0.05$ was adopted.

Results

Faecal pregnane assay validation

Serial dilutions of pooled faecal extracts from Svalbard and Finland samples showed parallel displacement with the standard curve (r = 0.97 for both) at $p < 0.01$ (Figure 1). The intra-assay CV was 4.50% and inter-assay CV's were 3.70% and 2.10% at 30% and 70% binding, respectively. The recovery of known concentrations of P4 (n = 6) was 113.10 ± 3.00% (mean ± SE). The measured hormone concentrations in the spiked samples correlated with the expected concentrations (r = 0.99; $p < 0.01$) (Figure 2).

Chromatographic separation of pooled faecal extracts revealed several major polar and non-polar peaks. Although peaks were not positively identified, coelution with peaks of the reference standards was used to determine the hormones or their metabolites. The immunoreactivities around fractions 28, 39, 57 - 60, and 64 correspond to the 20β-

progesterone, 17α-OH-progesterone, 20α- and 20β- dihydro-progesterone, and progesterone, respectively (Figure 3).

Relationship between plasma and faecal progesterone

We found a positive linear relationship between the log-transformed plasma P4 and faecal pregnane both in the Finnish herd ($b \pm SE = 0.892 \pm 0.046$, $p < 0.001$, $r^2 = 0.84$, Figure 4A) and in the Svalbard reindeer herd ($b \pm SE = 0.535 \pm 0.039$, $p < 0.001$, $r^2 = 0.74$, Figure 4B). The graphs also present separate groupings of the data points indicating a difference between low and high P4 and pregnane values.

Effect of pregnancy status and herd

In Svalbard, females pregnancy status was determined by ultrasound resulting in 56 pregnant females with plasma P4 concentration ranging from 3.58 to 14.13 ng/ml (mean ± SD = 7.87 ± 2.55) and corresponding faecal pregnane ranging from 2844.16 to 21064.31 ng/g (mean ± SD = 6643.56 ± 3937.92). Non-pregnant reindeer (n = 10) had a plasma P4 below 1.19 ng/ml (Figure 5A) and faecal pregnane below 1531.37 ng/g (Figure 5B).

In Finland, 18 females produced a calf (75% of all females) and had plasma P4 concentration ranging from 3.39 – 22.11 ng/ml (mean ± SD = 10.28 ± 4.20) and faecal pregnane ranging from 7270.81 – 19449.68 ng/g (mean ± SD = 11700.15 ± 2887.56). Non-pregnant reindeer (n = 40 including both sexes) had plasma P4 below 1.29 ng/ml (Figure 5A) and faecal pregnane below 2094.59 ng/g (Figure 5B).

Among 141 plasma samples that were analyzed from the two reindeer herds, a minimum cut-off value of 2.34 ng/ml P4 was found to maximize the accuracy of pregnancy diagnosis and a cut-off value of 2469.4 ng/g pregnane in dried faeces

maximized the accuracy of pregnancy diagnosis. As there were many possible cut-off values that gave 100% accuracy for pregnancy diagnosis, the median of these concentrations was chosen as the reference cut-off value. There was a significant difference between non-pregnant and pregnant female reindeer in both herds using both plasma P4 ($F_{1,112} = 303.71$, $p < 0.001$, Figure 5A) and faecal pregnane ($F_{1,112} = 361.48$, $p < 0.001$, Figure 5B).

In Svalbard, plasma P4 concentration ranged from 0.09 to 14.13 ng/ml (mean ± SD = 6.74 ± 3.57) and corresponding faecal pregnane ranged from 567.41 to 21064.31 ng/g (mean ± SD = 5783.12 ± 4164.42) in the 66 females sampled. In Finland, plasma P4 concentration ranged from 0.06 to 22.11 ng/ml (mean ± SD = 7.80 ± 5.60) and corresponding faecal pregnane ranged from 269.34 to 19449.68 ng/g (mean ± SD = 8941.29 ± 5381.19) in the 47 females sampled. We found that hormone levels differed between females of the herds even after controlling for the pregnancy status and after adding male data to the analysis ($F_{1,112} = 8.54$, $p = 0.008$ and $F_{1,112} = 21.40$, $p < 0.001$ for blood and faeces respectively, Figure 6).

Age class effect on progesterone levels

From the Finnish data, we found no difference between calves, males and non-pregnant females in terms of blood P4 ($p = 0.15$) or faecal pregnane ($p = 0.09$), even after removing the two non-pregnant females from the analysis. In Finland, plasma P4 levels did not differ between age classes for females ($p = 0.31$) while faecal pregnane did differ ($F_{2,46} = 5.53$, $p = 0.011$) with yearlings and adults grouped away from calves (Table 1). Male P4 did not differ between age classes for blood ($p = 0.56$) or faeces ($p = 0.58$) in Finland (Table 1).

In Svalbard, we found no significant difference between age classes when calf was in the model for plasma P4 ($p = 0.96$) or faecal pregnane ($p = 0.91$). When calves were removed from the analysis due to small sample size (n = 1), there again was no significant difference between yearling and adult females for plasma P4 ($p = 0.80$) or faecal pregnane ($p = 0.74$; Table 1).

Discussion

Relationship between plasma and faecal progesterone

For both Finland and Svalbard herds, we found positive relationships between plasma P4 and faecal P4 metabolite levels, which agree with studies by Capezzuto et al. (2008) in goats (*Capra sp*.) and by Shimizu (2005) on Japanese macaques (*Macaca fuscata*) who also found a strong relationship between blood and faecal P4. Faecal hormone analysis may therefore be a useful alternative to traditional blood endocrine studies including studies in reproduction, stress hormones, behavioural analysis, social ranking, and animal well-being (Cook et al. 2002, Rehbinder and Hau 2006). The performance of the pregnane assay used for faecal hormone analysis in this study was validated with parallelism, accuracy, and precision tests. These tests showed that the assay reliably measured the hormones present in the faecal extracts without being influenced by components within the extract.

Faecal pregnane may be influenced by other factors such as blood flow to the gut and liver, metabolic clearance rate, total faecal output, or lag time caused by the travel through the body (Cook et al. 2002); all of which could influence the strength of the relationship we witnessed. One issue to discuss when observing the relationship between plasma and faecal P4 is the apparent separation of lower and higher levels of P4 in the

plasma and faeces. In Figure 4, we noticed that there is a small range of samples with low P4 concentrations and then another group of samples with a wider range of higher P4 levels. This separation can be explained by the division between pregnant and non-pregnant animals.

Pregnancy cut-offs

We determined that reindeer with plasma P4 below 2.34 ng/ml should be considered non-pregnant while females with P4 levels above should be considered pregnant. Other studies have reported cut-off values between 1.6 ng/ml and 2.2 ng/ml for reindeer (Savela et al. 2009, Fjorden and Grontvedt unpublished). As for faecal pregnane, any value below 2469.4 ng/g from dried reindeer faeces should be considered non-pregnant, and anything above, considered pregnant. Progesterone metabolites, in particular, have been used to differentiate pregnant females from non-pregnant females or males for various species [e.g. caribou (Messier et al. 1990); free-ranging African elephants (Wasser et al. 1996); Sika deer (*Cervus nippon*) (Hamasaki et al. 2001); and Okapi (*Okapia johnsoni*) (Kusuda et al. 2007)].

Herd differences

There was a clear difference between the Finland and Svalbard herds for both plasma P4 and faecal pregnane, which can be attributed to a number of factors. On average, both plasma P4 and faecal pregnane concentrations were higher in Finland females than in Svalbard, however between individuals, variation was higher in Svalbard. It has been proposed that diet (both the type of food consumed and the digestive physiology) may have an influence on excreted P4 and should therefore be considered when comparing species, or in this case, sub-species (Ninnes et al. 2010). An increase of dietary fibre has

been shown to have a negative effect on progestogen excretion in captive baboons (*Papio cynocephalus cynocephalus*) (Wasser et al. 1993), but was found to have no effect on P4 metabolite concentrations in dairy cattle (Rabiee et al. 2002). As a general rule, healthy diets tend to increase plasma P4 turnover and animals produce less faeces, affecting both blood P4 and faecal pregnane concentrations (Cook et al. 2002). Svalbard reindeer feed solely on natural browse, while the Finnish reindeer were supplementary fed during the winter. It may be that the improved diet in the Finnish herd had an effect on the excreted pregnane, allowing the animals to produce faeces with a larger concentration of pregnane per pellet.

Higher pregnane concentrations found in Finnish reindeer may also be explained by differences in environmental conditions between herds. Svalbard reindeer are located much farther north (approximately 8° north of the Finnish herd) and are required to deal with a harsher winter climate (severe icing occurs frequently reducing access to food) (Fjorden and Grontvedt unpublished) for a longer period of time so perhaps P4 levels are affected. In colder, extreme conditions, a combination of energetic constraints such as poor natural forage, limited access to forage, and high metabolism, may require the overuse of body reserves and muscle proteins to survive. Poorer body health may have an effect on pregnane (Cook et al. 2002, Taillon and Côté 2008). During winter, birds having reduced body condition and survival probability had higher T concentrations (Ketterson et al. 1991). Studies have found that pregnancy success may also be related to female body weight and age (Reimers 1983), therefore reduced body condition may directly affect P4 and subsequently the success of pregnancy. In herds of semi-domesticated reindeer, such as our Finnish study herd, yearlings often become pregnant,

demonstrating that the reproductive process occurs earlier in their life. In the high arctic Svalbard herd, yearlings are rarely pregnant (18.8% pregnancy rate as yearlings versus 81.8% pregnancy rate near their third year or older), most likely due the stressful environmental conditions (Ropstad 2000).

Age class effect on progesterone levels

We found no significant difference between blood or faecal P4 concentration levels of calves, adult non-pregnant females and males. This signifies that if an animal was to have < 2.34 ng/ml plasma P4 or < 2469.4 ng/g dried faecal pregnane, we cannot be certain whether the animal is an adult female that is not pregnant, a male, or a calf of either sex, making the use of P4 levels as a tool in the field to differentiate between calves, males and non-pregnant females unreliable. In Finland, there was a significant difference found in faecal pregnane concentration levels between females of different age classes, indicating that faeces may provide a distinction between calves and older female reindeer (yearlings and adults). Reindeer in Svalbard and male reindeer in Finland, on the other hand, could not be separated into age classes using blood P4 or faecal pregnane levels. Endocrine physiology is generally different between young, adolescents, and adults which may affect the concentrations of hormones that influence physiological processes such as growth, immune and reproductive functions (Seraphin et al. 2008). However, P4 is mostly secreted by the corpus luteum and placenta for pregnancy maintenance, with limited amounts being generated by the adrenal gland (Plotka et al. 1983), and therefore does not seem to differ between age classes but rather depends on the reproductive status of the individual (Flood et al. 2005).

Management Implications

We documented that faecal progesterone metabolites, specifically pregnane, reflect the levels of circulating P4, and can therefore be used to monitor and manage animal populations. Therefore, we recommend using non-invasive faecal sample collections during the winter as a technique to monitor reproductive status of the sub-species *Rangifer tarandus*. Unfortunately, there are limitations to the use of faecal pregnane to predict certain population parameters such as age class and sex. However, since sex can be differentiated using faecal DNA techniques (e.g. Wasser et al. (1997)), and that faecal pellet size has proven promising for age class identification (e.g. Ball (2010), Chapter 2 in this thesis), a combination of hormone, DNA, and pellets morphometry would be a feasible combination of non-invasive techniques for population monitoring. Faecal DNA may provide individual identification, sex, and an estimation of the population size using capture-mark-recapture methods (Hettinga 2010). Once identification has been determined, the faecal pregnane and pellet size may also provide information on age class and reproductive status, therefore, improving our understanding of the population dynamics.

Table 2. Least square means (lsmeans ± SE) of plasma progesterone (ng/ml) and faecal pregnane (ng/g) concentrations levels, adjusted for pregnancy status and month, in Finland and Svalbard, for various age classes (C = Calf, Y = Yearling, A = Adult). Means with the same letter are not significantly different according to a "Tukey-Kramer" multiple comparisons test ($\alpha = 0.05$).

	Sex	**Hormone**	**Age class**		
			C	**Y**	**A**
Finland	F	Log(plasma)	0.051 ± 0.180	0.454 ± 0.145	0.508 ± 0.157
		Log(faeces)	3.152 ± 0.095^{a}	3.597 ± 0.077^{b}	3.666 ± 0.083^{b}
	M	Log(plasma)	-0.354 ± 0.121	-0.478 ± 0.121	-0.298 ± 0.113
		Log(faeces)	2.759 ± 0.098	2.648 ± 0.098	2.622 ± 0.091
Svalbard	F	Plasma	-	3.796 ± 1.482	4.231 ± 0.517
		Log(faeces)	-	3.334 ± 0.119	3.380 ± 0.041

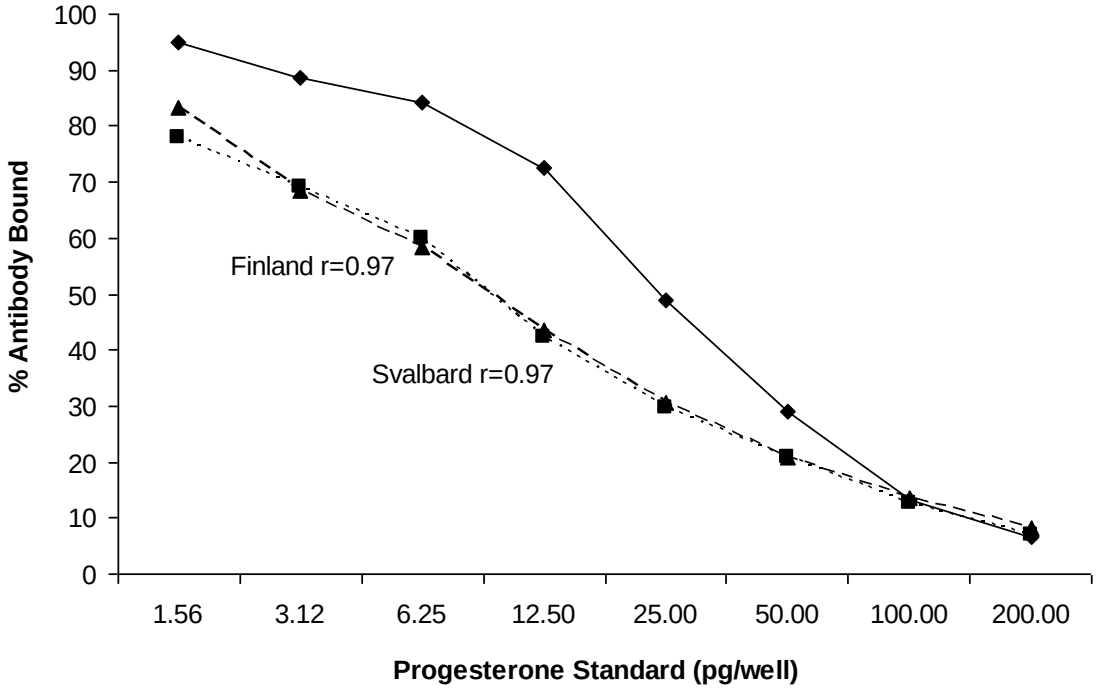

Figure 1. Parallelisms for serial dilutions of pooled faecal extracts of Svalbard and Finland samples against the standard curve. Solid diamond (♦), standard curve; dotted square (■), Svalbard pooled faecal extract dilutions; dashed triangle (▲), Finland pooled faecal extract dilutions.

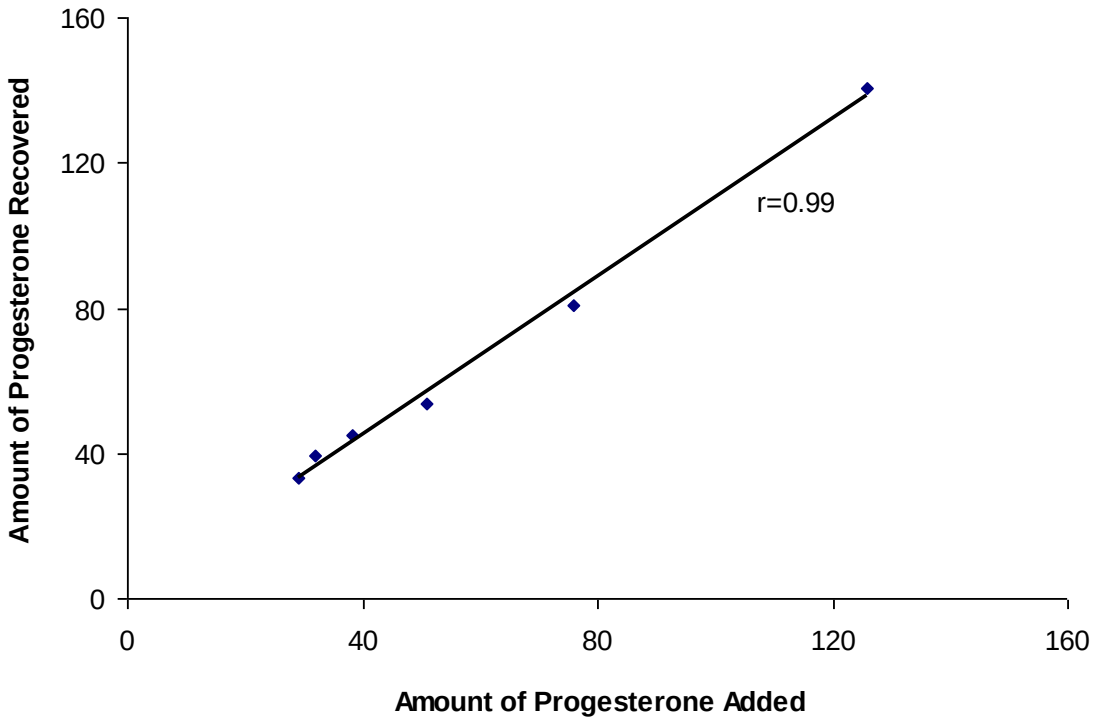

Figure 2. Recovery of exogenous hormones from pooled faecal extracts.

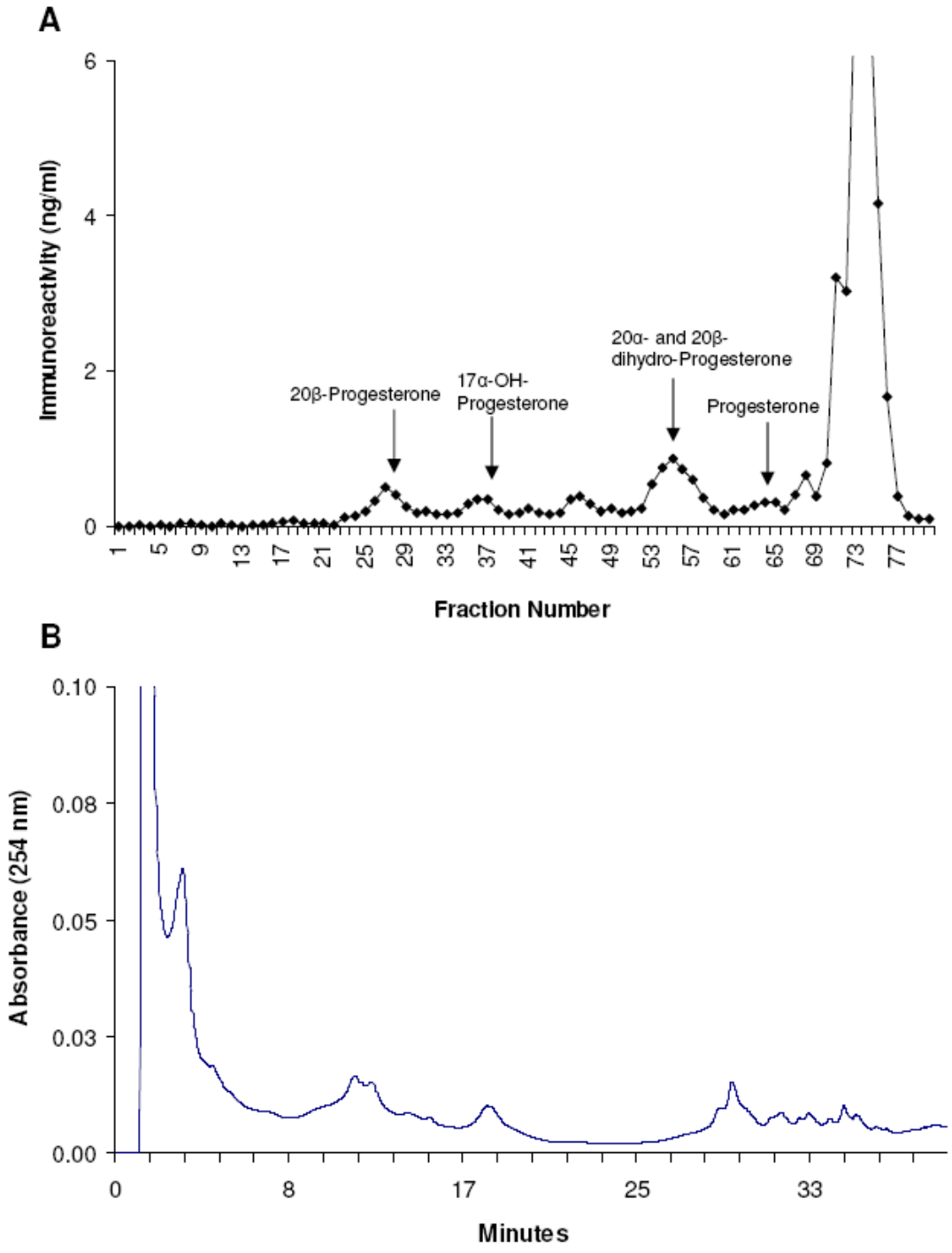

Figure 3. HPLC separation of pooled faecal extracts. Immunoreactivity as determined by EIA (A) and HPLC elution profiles (B) are presented.

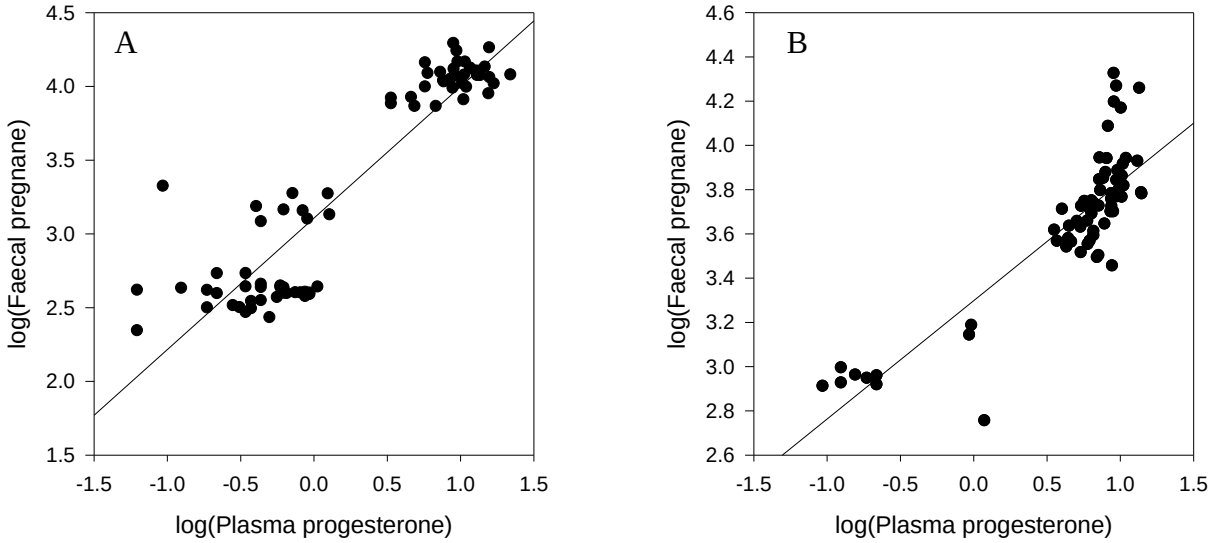

Figure 4. Relationship between log-transformed faecal pregnane and log-transformed plasma progesterone concentrations in (A) the Finland herd and (B) the Svalbard herd.

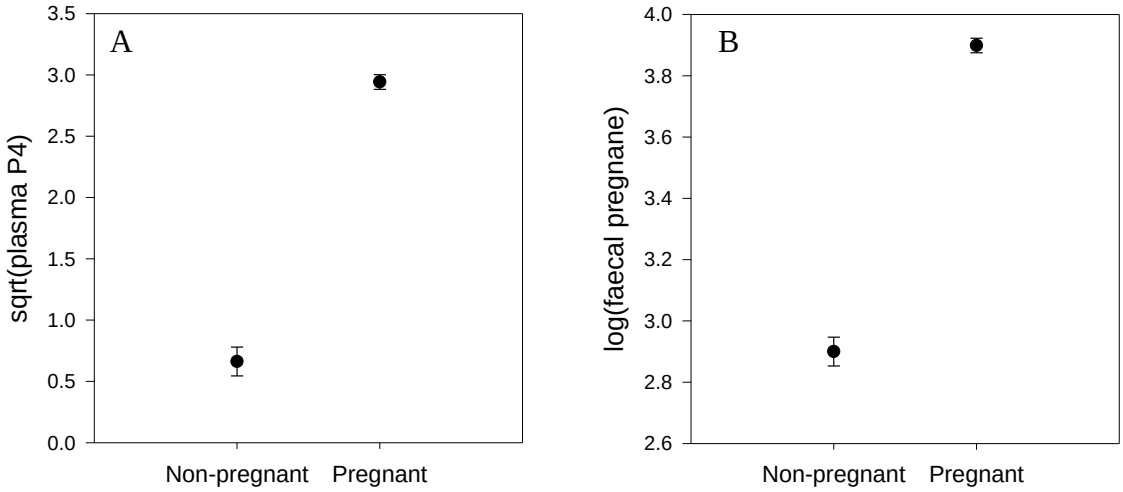

Figure 5. Difference between non-pregnant and pregnant reindeer in Finland and Svalbard herds in (A) square-root transformed plasma progesterone and (B) log-transformed faecal pregnane concentrations (lsmeans ± 1 SE).

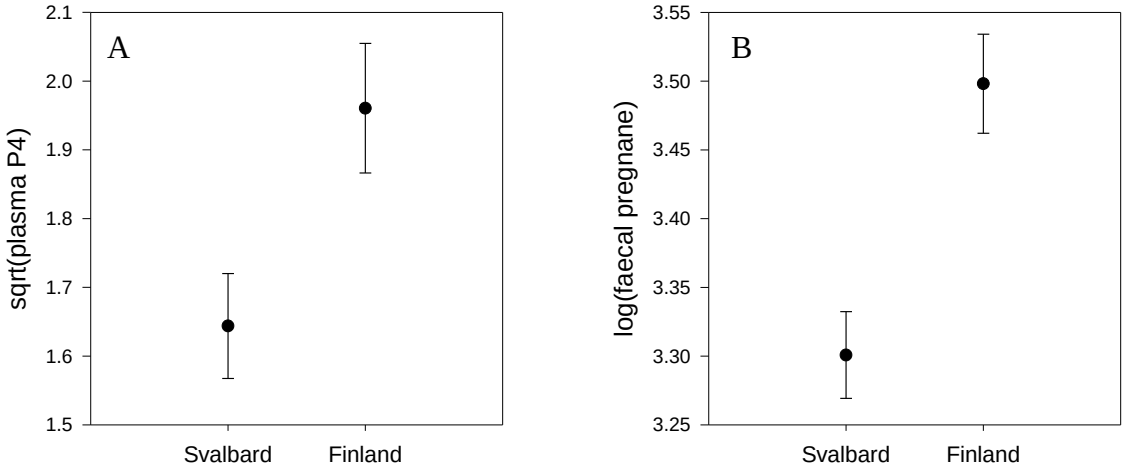

Figure 6. Difference between Finland and Svalbard herds in (A) square-root transformed plasma progesterone and (B) log-transformed faecal pregnane concentrations (lsmeans ± 1 SE).

Chapter 2:

Use of faecal pellet size to differentiate age classes in reindeer and caribou

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Abstract

Proper management of threatened populations requires prior knowledge of population sizes and structures, however current techniques to gather this information are generally impractical, costly, and can be physically stressful for the animals. Non-invasive methods (e.g. faecal sampling) that can produce high quality and accurate results are better alternatives. Using faecal samples collected from a Svalbard reindeer population in winter 2008 (n = 158) and 2009 (n = 161), we investigated and validated the feasibility of using faecal pellet sizes to differentiate between calves, yearling and adult females. We found that pellets from adults were longer than those from calves; and pellets from adults and yearlings were clearly wider than those from calves. With an accuracy of 91% correct classification, we did show that a combination of faecal pellet dimensions (length, width, and depth), rather than a single dimension alone, can allow managers to clearly differentiate between age classes. We also found a positive relationship between live weight and pellet size of the reindeer. This information may provide important population parameters and be a valuable tool for the monitoring of threatened populations of woodland caribou, as well as wild reindeer.

Introduction

Knowledge of demographic parameters is essential for monitoring populations of wild animals, especially threatened species. Management of wild populations requires more information than indices of abundance or density; it also requires knowledge of the age and sex composition (Mysterud & Ostbye 2006), as such data may be used to predict future population dynamics. For example, under low hunting risk, a population composed of more than 15% of caribou calves would be indicative of growth (Bergerud 1992); thus knowledge of the age composition of the population is essential in predicting future population trends. Unfortunately, techniques to estimate population structures of certain species, especially those that are elusive and rare, are generally impractical and costly (Haigh 1979, Valkenburg et al. 1983) and have been criticized as being harmful to the animals (Côté et al. 1998, Cattet et al. 2008, Omsjoe et al. 2009). It is, therefore, in our best interest to seek out methods that are economically practical and safe, yet can still produce similar high quality, accurate results. Non-invasive monitoring techniques, for example DNA microsatellites, sex hormones, and faecal pellet morphology are currently being studied to replace the more risky alternatives (Southgate 2005).

Population dynamics of ungulate populations depend not only on total numbers and densities, but also on sex and age structures; therefore proper management and prediction of future dynamics require proper estimates of these variables (Mysterud & Ostbye 2006). Indeed, in many ungulates, the population structure will affect their population dynamics (Coulson et al. 2001, Gaillard et al. 2003). Knowledge of the age structure of a population is essential for exploring trends in recruitment, population growth, mortality, and reproductive status of the population; all which may contribute to

better monitoring and management of the species (Reilly 2002). An increase in age is linked to variation in performance levels; an older population may have more senescent behaviours than a prime-aged or sub-adult population (Mysterud & Ostbye 2006, Weladji et al. 2010), therefore it is essential to know the age structure of a population to understand future trends.

Field biologists often spend substantial time and money to capture and tag young animals for the purpose of identifying their age later in life (Delibes-Mateos et al. 2009). Common age estimation tools include tooth sectioning, molar tooth wear, eye lens weight, the measurement of different body sections, the progression of lumbar epiphyseal fusion, and body weight; all of which often require the capture or killing of some animals, which is not always possible or good for population viability (Delibes-Mateos et al. 2009). Another common tool for age determination is by visual estimation during helicopter surveys, however, the accuracy of this method requires that the animals be in open areas and therefore may not be useful for woodland caribou (Miller 2003, Reimers 2006). It has been argued that faecal pellet size can be used as a non-invasive technique to identify age classes since individual animals can be difficult to capture, but faecal pellets are relatively easy to find and collect (Southgate 2005). Many studies have attempted to use pellet size or dimensions such as diameter, weight, or volume of the pellets to predict age (MacCracken & Van Ballenberghe 1987, Reilly 2002, Chapman 2004, Southgate 2005). However, most of these studies have used only one measure of pellet size or dimension which may work for some species, but more often make age classification unclear due to pellet size overlap. Accordingly, we argue that a combination of dimensions may be a more promising approach. Indeed, Khan and Goyal

(1993) concluded that one pellet measurement alone was not sufficient to predict the age of an individual, however a combination of length, maximum width, and weight measurements were able to predict the age in female Manipur Brow-Antlered deer (*Cervus eldi eldi*).

Woodland caribou (*Rangifer tarandus caribou*) populations are declining in much of their natural range across North America and is listed as threatened by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC 2002). Not all populations of woodland caribou are monitored, in fact in a review by Edmonds (1991), of the 44 herds she was studying, 50% of the populations had an unknown status. Since the cost of monitoring is high and techniques to gather necessary data are often invasive, the appeal of using faecal pellet size as a non-invasive tool to monitor threatened caribou populations is great.

Faecal pellet size has also been reported to be correlated with both body mass and age in several species. For example, Uye and Kaname (1994) found a strong correlation between body size and their faecal pellet size of zooplankton, with larger zooplankton excreting larger pellets. Putnam (1984) also reported that within a given species, that weight of individual dung pellets may be related to the weight of the individual defecator; thus allowing an age-structure to be created. Since *R. tarandus* body size increases as the individual ages to adulthood, there is potential that pellet size may indicate the sex and age structure of a population (see e.g. Bubenik (1982) for elk *Cervus elaphus*, MacCracken and Van Ballenberghe (1987) for moose *Alces alces*). However, this remains to be investigated for *Rangifer* species.

Since woodland caribou populations are wild in much of northern Canada, there are no suitable study populations where individual ages are known. Therefore, we used the Svalbard reindeer (different subspecies) herd that has been monitored since 1994 to collect faecal data from individuals of known age and body mass. In this paper, we investigated the possibility of using winter faecal pellet groups to identify age class of female Svalbard reindeer based on pellet morphometry (either single dimension or combination of measurements) and validated our model using data from another year from the same population. This permitted the development of a tool to estimate the age structure of a wild population of woodland caribou in the future. Further, we assessed the relationship between faecal pellet morphometry and body size, as a possible explanation for faecal size differences that may exist between age classes.

Materials and Methods

Study Area

The study took place in Nordenskiøldland, Spitsbergen (77°50`–78°20` N, 15°00`–17°30` E), Svalbard in Norway, including Colesdalen, Semmeldalen and parts of Reindalen, with adjacent side valleys. The area is home to a population of about 1500 Svalbard reindeer *Rangifer tarandus platyrhynchus* (Milner et al. 2003). Human presence is rare and predators are limited, so this wild population's only threat is the extreme environment that often causes large fluctuations in population sizes (Meteorologisk institutt 2009). Most reindeer feed on lichens during the winter, however Svalbard reindeer are limited to feeding on browse of mosses and low-quality vascular plants which are often difficult to find and attain (Bjorkvoll et al. 2009, Sundset et al. 2009). Since 1994, female reindeer have been caught and sampled once a year for

individual survival and reproductive rates, however it has only been since 2007 that the animals have been caught in both February and April, coinciding with increased sampling protocols (Fjorden and Grontvedt, unpublished data). Because it is a long-term, closely studied population, a large percentage of the females are marked with ear tags and neck collars for identification and are of known age (Milner et al. 2003).

Data Collection

Methods of capture are described by Milner et al. (2003). Faecal and blood samples were collected from individual reindeer during four visits to Svalbard, during February and April of 2008 (n = 158) and 2009 (n = 161). The faeces were either gathered directly from the anus or from the snow immediately following excretion. The faecal and blood samples remained frozen at –20° C until analysis. The study was carried out in agreement with the provisions enforced by the Norwegian National Animal Research Authority (NARA). All activities related to capturing and sampling animals were done with permission from the authorities of Svalbard. We collected 319 different pellet groups from 272 female Svalbard reindeer (1834 pellets total), 263 pellet groups from adults, 25 pellet groups from yearlings, and 31 pellet groups from calves.

Using the running mean approach, we found that five random pellets per individual was an adequate sample size for the analysis. Each pellet was measured for its maximum length (L), maximum width (W), and depth (D) at 90° rotation from W (Figure 7). Only complete, regular oval pellets were used (Zahratka & Buskirk 2007). To avoid any inter-observer bias, all samples were measured by the same observer using Vernier calipers with precision of 0.05 cm (Sanchez-Rojas et al. 2004). An approximated volume

index (V) was calculated using the product of the three measurements (L × W × D). We obtained the mean value for each measurement per animal per sampling period.

Statistical Analysis

We used a generalized linear mixed model [PROC MIXED using the SAS 9.2 software; SAS Institut Inc. 2008] to explore the relationship between the collection periods (2 levels: February and April) and the age classes [3 levels: calves (0-1 yr; C), yearlings (1-2 yrs; Y), and adults (2+ yrs; A)] of pellet sizes using 2008 data. "Individual ID" was included in our model as a random factor to account for repeated measurement on the same individuals, thereby controlling for pseudoreplication. There is often controversy with the development of ecological indices and their effectiveness in reality; therefore it is essential to carry out case-specific verification and validation for each species (Delibes-Mateos et al. 2009, Rouco et al. 2009). We therefore performed a cross-validation using a subsample of our data (Conroy & Carroll 2009). Unfortunately, due to severe weather icing conditions in the winter of 2008 on Svalbard, high calf mortality occurred, therefore samples collected in 2009 lacked any calf samples and very few yearling samples. To deal with this issue, we limited our mixed model analysis to data from February and April 2008 using a random subset of 20 calves (65% of all calves) generated by Minitab 15.0 (Minitab Inc. 2007) to analyze the pellet size data. We used the second year of data, from 2009, and the remaining 11 random calves to validate our model and to see how accurately age class can be predicted in the second year by various pellet morphometry models.

We also used a multiple discriminant analysis (MDA) [PROC DISCRIM using the SAS 9.2 software; SAS Institute Inc. 2008] to investigate the difference between groups,

specifically the three age classes (Ims & Yoccoz 2000). Multiple discriminant analysis is used to classify grouping dependent variables which have two or more categories, in our case age class, using multiple predictors variables such as pellet dimensions (Sanchez-Rojas et al. 2004, Garson 2008). The accuracy of the classification function was estimated as the number of animals correctly classified depending on their mean pellet size (MacCracken & Van Ballenberghe 1987). We therefore assessed all possible models to determine which most accurately identified the correct age class.

To assess the effect of age class (calves, yearlings, adults) on body mass of female reindeer, we also used linear mixed model, with "individual ID" entered as a random term and the weighing date and year being entered as covariates to account for change on body mass with time. From these models, we generated least square means (lsmeans) and the corresponding standard error for each age class. Finally, we used linear mixed models to assess the relationship between live body weight and the mean dimensions (i.e. average of the 5 samples per individual) of the faecal pellets (Zahratka & Buskirk 2007), the effect of "year" and "month" being controlled for and "individual ID" being added as a random term. All the analyses were performed using SAS 9.2 software (SAS Institute Inc. 2008) and a significance level of 5% was adopted.

Results

Age class versus pellet size

Adult pellets were longer than those from calves (Estimated difference: A-C = 0.146, SE = 0.044) and there was no difference in length between pellets from adults and yearlings as well as calves and yearlings (Table 2A, Figure 8A). Adult and yearling pellets were wider than calf pellets (Estimated differences: A-C = 0.119, SE = 0.018 and Y-C = 0.059,

SE = 0.024), additionally adult pellets were wider than yearling pellets (Estimated differences: A-Y = 0.060, SE = 0.019) (Table 2B, Figure 8B). Adult pellets were deeper than both calf (estimated difference: A-C = 0.108, SE = 0.017) and yearling (estimated difference: A-Y = 0.064, SE = 0.018) pellets, but there was no difference between calf and yearling pellets depth (Table 2C, Figure 8C). Finally, the volume of adult pellets was greater than those of both calves (estimated difference: A-C = 0.286, SE = 0.052) and yearlings (estimated difference: A-Y = 0.160, SE = 0.054) but there was no difference in volume between calf and yearling pellets (Table 2D, Figure 8D).

Using validation and cross-validation techniques, single dimensions were able to correctly classify pellets into age classes with a range of accuracy of 56.4% for length alone to 79.1% for depth or width alone. A combination of length and depth was able to correctly classify 89% of the pellets into age classes, but the most accurate combination of dimensions included length, width, and depth with an accuracy of 90.7% of correct classification into age class.

Results of MDA revealed the best model for overall accuracy to be the length and width combination with an error of 24.4%. We also found that the combination of length, width, and depth only provided intermediate accuracy with an error of 25.7%. Focusing solely on the correct assignment of the calf age class, we found that both the length and width model and the length, width, and depth model were the most accurate with 80.7% accuracy (Table 3).

Body mass versus pellet size

We found body mass to differ between calves, yearlings and adults female reindeer ($F_{2,116}$ = 210.05, $p < 0.001$), the smallest animals being the calves (lsmeans = 29.17 kg, SE =

0.96) followed by the yearlings (lsmeans = 38.64 kg, SE = 0.96) and adults being the largest (lsmeans = 49.50 kg, SE = 0.37). We also found a positive relationship between live weight and all pellet size dimensions (length: b = 0.005, SE = 0.001, R^2 = 0.081, Figure 9A; width: b = 0.005, SE = 0.001, R^2 = 0.492, Figure 9B; depth: b = 0.005, SE = 0.0004, R^2 = 0.507, Figure 9C; volume: b =0.014, SE = 0.001, R^2 = 0.420, Figure 9D; Table 4). Length, width, depth and volume did not differ between years, while the effect of month was only significant for depth (Table 4).

Discussion

Age class versus pellet size

We investigated the use of pellet size (pellet length, width, depth, and volume) as a method for classifying reindeer into major age categories (calves, yearlings and adults). Generally, the highest values across all of the variables occurred in adult samples, yearlings were intermediate, and calves had the smallest values, similar to what was found in mule deer (Sanchez-Rojas et al. 2004). Overall, we found that pellet size differed significantly between age classes; however one dimension alone was not sufficient to clearly differentiate among age classes. Indeed, using a second year of data, we were also able to validate various models and found that the best model to predict age class most accurately, specifically in differentiating calves from other age categories, was a combination of length, width, and depth of the pellets with an accuracy of 91%. Several studies have concluded that pellet size alone was not able to correctly group the pellets by age class; however these studies limited their research to simple models, using few, single pellet dimensions rather than combining multiple measurements (Chapman 2004, Southgate 2005, Delibes-Mateos et al. 2009). Southgate (2005) was able to

differentiate immature-independent individuals (<500 g) from larger females and males (+500 g) by using the faecal pellet diameter of the greater bilby (*Macrotis lagotis*). Overall, we found that adults could be more clearly separated from the other two age classes based on pellet dimensions as calf and yearling pellet sizes were more variable, similar to what was found in moose (MacCracken & Van Ballenberghe 1987).

The model that was the most accurate for data grouping, as determined by the discriminant analysis, was the combination of length and width measurements. However, using the validation accuracy from the second year of data, only 86% pellet groups were correctly identified compared to 91% accuracy found with the length, width, and depth model. The discriminant analysis suggested that either length and width together or the length, width, and depth model could be used to most accurately separate the calf age class (81% correct identification) from yearling or adult reindeer (Table 3). Therefore, we believe that the combination of all three measurements; length, width, and depth of the pellet groups will provide a good basis on which to separate age classes in Svalbard reindeer, and perhaps other ungulates species or subspecies such as woodland caribou. It seems that the accuracy of using pellet size data (simple or complex models) to determine age class is highly dependent on the species under investigation.

Body mass versus pellet size

We found that pellet size as well as body mass differed between calves, yearlings and adult; being higher for adults and smaller for calves. We also found a positive relationship between female body mass and pellets size (Figure 9). Calves were on average nearly 20 kg lighter than adult reindeer. Many other studies have suggested that as the study species ages or grows in size, faecal pellet size and weight will also increase

(Simonetti & Fuentes 1982, Chapman 2004, Sanchez-Rojas et al. 2004). Coe and Carr (1983) found a highly significant linear relationship between ungulate body weight and dry weight of faecal pellets. However, Southgate (2005) only found a weak relationship between pellet diameter and animal weight in the greater bilby. Our faecal pellet size data also showed a positive relationship with live weight (Table 4). There was strong evidence that width, depth, and volume of faecal pellets are well predicted by body weight, and therefore may be able to help predict age class (Zahratka & Buskirk 2007).

Using faecal pellet morphometry, we show that it is possible to differentiate between calf, yearling, and adult female reindeer. Although we only used data from one Svalbard reindeer population, we believe it would be possible to apply these same procedures and models to other *Rangifer* populations elsewhere, including woodland caribou (Ball et al. 2010). Unfortunately, Svalbard reindeer are much smaller than most other subspecies of *R. tarandus*; the average body weight for a female *R. t. platyrhynchus* is 38.0 ± 6.0 kg (± SD), which is significantly less than *R. t. tarandus* with the average body weight measuring 63.1 ± 10.0 kg (Ropstad et al. 1999). Given that body weight is strongly correlated to faecal pellet size, we may either scale up our dimension ranges using, for example, the ratio of body mass of two subspecies or their allometric relationships.

During our analysis, we often found that the month of sampling influenced variation in our dimensions (Table 2). It may therefore be important in the future to examine the influence of seasonal changes in diet on faecal pellet size, a problem which has been raised by several authors (Bhadresa 1982, MacCracken & Van Ballenberghe 1987, Khan & Goyal 1993, Alvarez 1994, Sanchez-Rojas et al. 2004, Delibes-Mateos et

al. 2009). On Svalbard, the reindeer are free-ranging and feed on natural vegetation, therefore we would not expect a significant difference in feeding behaviors within the species, especially between two winter months depending on the weather. Frequently, animal species have behavioral attributes dependent on their age. Nutritional and energy requirements can be different depending on the age of the individual. Perhaps behavioral differences in reindeer or woodland caribou between age classes will affect diet and therefore may be attributed to the size difference seen in pellet morphometrics.

Another direction that could be explored is to examine the effect of decay and age of the pellet on the accuracy of age class estimation. In several studies the pellets were dried prior to measurement, however we would expect that if the animals were free-ranging, the water content of the pellets would not vary to a large extent as they have similar diets (Coe & Carr 1983, MacCracken & Van Ballenberghe 1987, Khan & Goyal 1993, Reilly 2002, Sanchez-Rojas et al. 2004, Delibes-Mateos et al. 2009). One option would be to develop a correction factor for the estimation of water content in the pellets (Coe & Carr 1983). However Reilly (2002) found that drying the dung was not necessary and that there wasn't a great enough effect of decay on the dung size of elephants to make it worthwhile, but perhaps in reindeer, there may be an effect even if the samples are frozen.

Management Implications

We here show that a combination of pellet measurements can be used to differentiate between calves, yearlings, and adult Svalbard reindeer. The dimensions we found most important to measure were a combination of maximum length, maximum width, and depth. We hope that pellet size-age class data, in combination with DNA and hormone

techniques, may improve the current non-invasive techniques that are used to monitor wild populations of woodland caribou and reindeer. Reilly (2002) found that dung data was more reliable to estimate age and population size than direct observations of elephants, which we hope will also prove to be for *Rangifer* spp. Faecal pellet data has several benefits in wildlife management, mainly lowering the cost of monitoring programs. It is a non-invasive procedure causing no harm to the animals and it may provide us with valuable information on population dynamics, such as recruitment rate, to help offer glimpses into the future (Reilly 2002, Sanchez-Rojas et al. 2004).

Table 2. Results of the mixed models assessing the effect of age class on pellet length (a), width (b), depth (c) and volume (d) of Svalbard reindeer in 2008. The effect of month (entered as a class variable with two levels, February and April, the latter being the reference) was controlled for and individual ID was entered as a random factor. Age class was entered as a class variable with 3 levels, adults, yearlings and calves, with yearling as the reference level. Significant terms are shown in bold face.

	b	SE	*t* (Df = 20)	*p* value
a) Length (cm)				
Intercept	1.340	0.045	29.86	≤0.001
Age class : Adult - Yearling	0.036	0.046	0.80	0.435
Calf - Yearling	-0.110	0.059	-1.86	0.078
Month: February - April	0.048	0.029	1.67	0.110
b) Width (cm)				
Intercept	0.729	0.019	39.37	≤0.001
Age class : Adult - Yearling	0.060	0.019	3.16	**0.005**
Calf - Yearling	-0.059	0.024	-2.43	**0.025**
Month: February - April	0.039	0.011	3.42	**0.003**
c) Depth (cm)				
Intercept	0.685	0.018	39.08	≤0.001
Age class : Adult - Yearling	0.064	0.018	3.56	**0.002**
Calf - Yearling	-0.044	0.023	-1.92	0.069
Month: February - April	0.023	0.010	2.34	**0.030**
d) Volume (cm^3)				
Intercept	0.666	0.053	12.67	≤0.001
Age class : Adult - Yearling	0.160	0.054	2.99	**0.007**
Calf - Yearling	-0.126	0.069	-1.82	0.084
Month: February - April	0.108	0.034	3.17	**0.005**

Table 3. Discriminant analysis for Svalbard reindeer pellet groups showing the two potential grouping models. First number is actual number of pellet groups classified into representative age class. Number in parentheses is % of pellet groups classified into representative age class.

	Classified as		
	Calf	Yearling	Adult
Model 1: L × W			
Calf	25 (80.65)	4 (12.9)	2 (6.45)
Yearling	6 (24)	18 (72)	1 (4)
Adult	18 (6.84)	50 (19.01)	195 (74.14)
Model 2: L × W × D			
Calf	25 (80.65)	4 (12.9)	2 (6.45)
Yearling	7 (28)	17 (68)	1 (4)
Adult	17 (6.46)	51 (19.39)	195 (74.14)

Table 4. Results of the linear mixed models assessing the relationship between live weight of females and pellets length (a), width (b), depth (c) and volume (d) of Svalbard reindeer. The effect of month (entered as a class variable with two levels, February and April, the latter being the reference) and year (entered as class variables with two levels, 2008 and 2009, the latter being the reference level) was controlled for. Significant terms are shown in bold face.

	b	SE	*t* (Df = 55)	*p* value
a) Length (cm)				
Intercept	1.118	0.055	20.21	≤0.001
Live weight	0.005	0.001	4.90	**≤0.001**
Year: 2008 - 2009	0.042	0.024	1.78	0.080
Month (February - April)	-0.034	0.022	-1.57	0.122
b) Width (cm)				
Intercept	0.565	0.025	23.05	≤0.001
Live weight	0.005	0.001	11.10	**≤0.001**
Year: 2008 - 2009	-0.000	0.011	-0.02	0.983
Month (February - April)	-0.003	0.009	-0.29	0.773
c) Depth (cm)				
Intercept	0.528	0.021	24.86	≤0.001
Live weight	0.005	0.000	12.50	**≤0.001**
Year: 2008 - 2009	0.004	0.009	0.44	0.664
Month (February - April)	-0.019	0.008	-2.53	**0.015**
d) Volume (cm^3)				
Intercept	0.232	0.066	3.49	0.001
Live weight	0.014	0.001	10.59	**≤0.001**
Year: 2008 - 2009	0.027	0.029	0.95	0.345
Month (February - April)	-0.035	0.026	-1.35	0.182

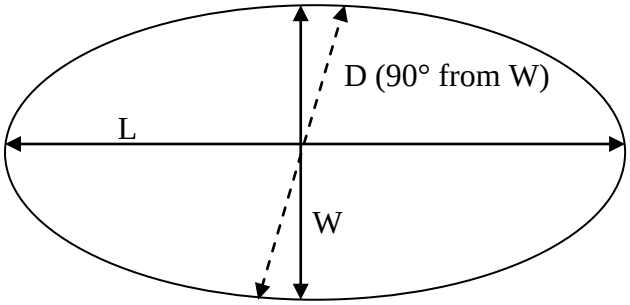

Figure 7. Sketch graph of the faecal pellet dimensions. (L stands for length, W for width, and D for depth (depth is measured at 90° from the width)).

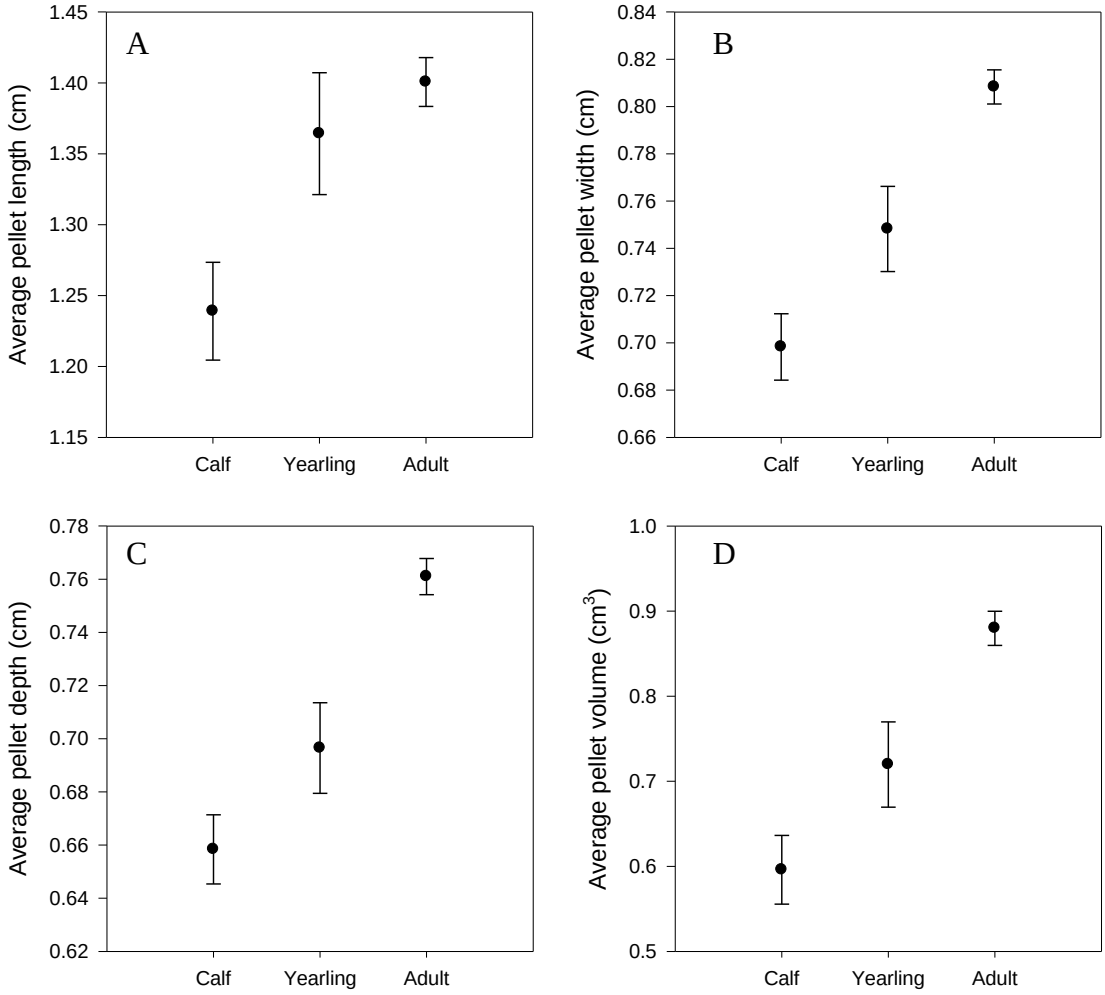

Figure 8. Least square means (adjusted for month) of pellets length (A), width (B), depth (C), and volume (D) as a function of the three age classes for Svalbard reindeer using a random set of calves. Bars are ± 1 SE.

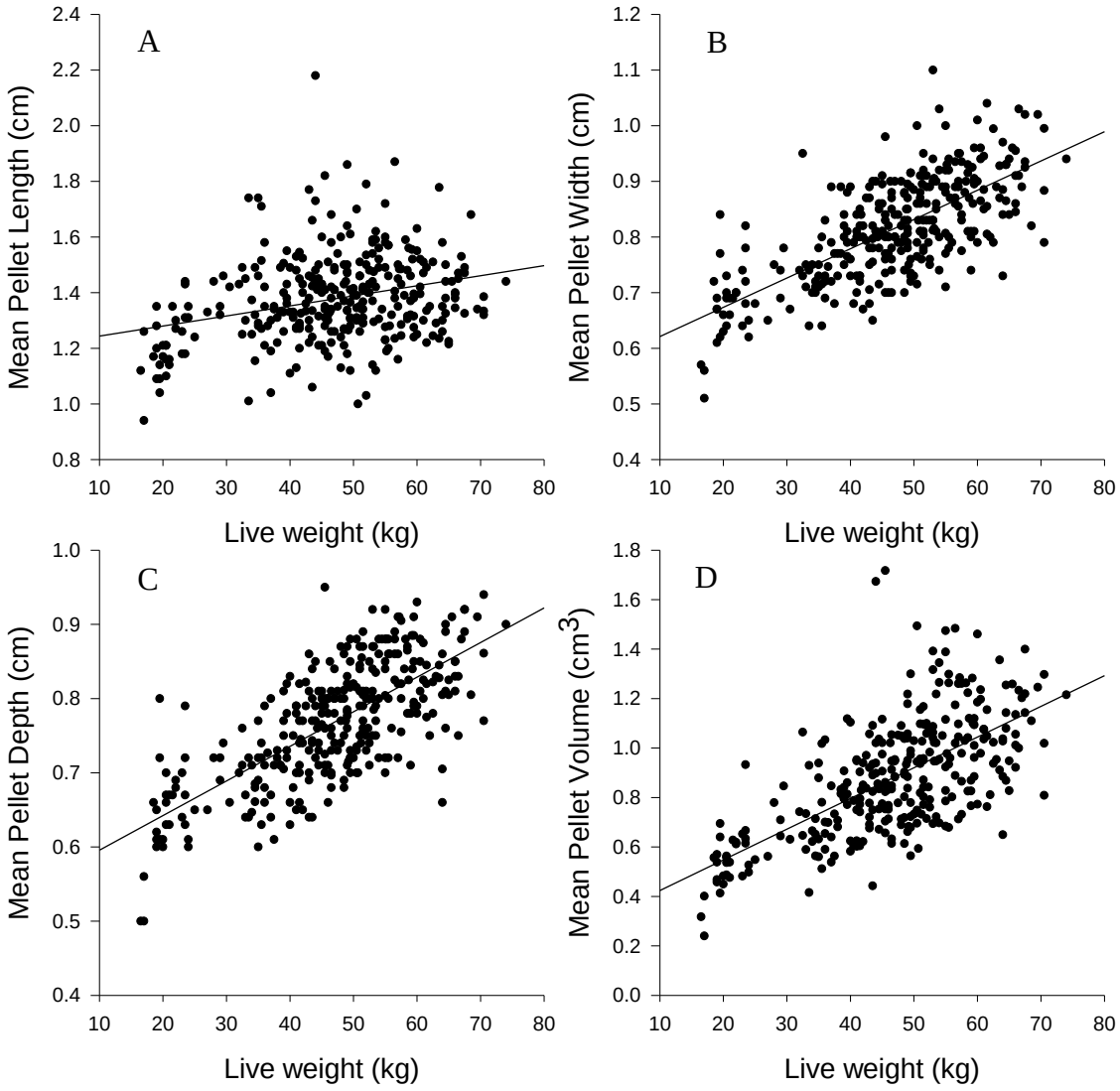

Figure 9. Linear regression of pellet length (A), width (B), depth (C), and volume (D) and Svalbard reindeer live weight (kg).

General Discussion

This study evaluated the potential of using faecal sampling from reindeer or caribou as a possible non-invasive population monitoring technique. I explored the possibility of using either or both faecal sex hormones, specifically progesterone metabolites, and faecal pellet size to estimate important individual and population parameters namely reproductive status, sex, and age in Svalbard and Finnish reindeer. The three first objectives are addressed in Chapter 1, while the last one is covered in Chapter 2. We found that faecal pregnane was strongly related to the concentration levels of plasma progesterone therefore allowing us to use faecal pregnane as a substitute for blood sampling, which is consistent with other studies (e.g. Capezzuto et al. (2008) in goats (*Capra sp*.) and Shimizu (2005) on Japanese macaques (*Macaca fuscata*)). Hormones found within the circulation are considered only a "snap-shot" in time, therefore any situation causing hormone fluctuations i.e. capture stress, will be seen directly in the blood (Ninnes et al. 2010). Faecal hormones, on the other hand, are cumulative overtime so changes in hormone concentrations tend to be more subtle and are more reliant on diet and environmental conditions (Wasser et al. 1993, Taillon and Cote 2008, Ninnes et al. 2010).

We also hoped to find that faecal pregnane could be used for pregnancy diagnosis in reindeer. Using ultrasound and observation of calf at heel for validation, we determined pregnancy status using both plasma progesterone and faecal pregnane cut-off concentrations. We found that it was possible to determine pregnancy using faecal pregnane alone with a cut-off of 2469.4 ng/g for both herds. Faecal progesterone has been a successful indicator of pregnancy in several other species (e.g. caribou (Messier et

al. 1990); free-ranging African elephants (*Loxodonta africana*) (Wasser et al. 1996); Sika deer (*Cervus nippon*) (Hamasaki et al. 2001); and Okapi (*Okapia johnsoni*) (Kusuda et al. 2007)) and now including in two sub-species of reindeer (this study).

We also examined the use of both plasma progesterone and faecal pregnane to estimate age class in female and male reindeer. We found that faecal pregnane differentiated calves from the two older age classes (yearlings and adults) for females in Finland, but not in Svalbard. We determined that a larger sample size per age group may help reduce some of the error, but since progesterone is directly linked to pregnancy (Flood et al. 2005), faecal pregnane may not be the best indicator for estimating age but rather be limited to pregnancy diagnosis. Other sex hormones such as testosterone have had more success in identifying age of an organism (Lynch et al. 2002, Rooney et al. 2004, Castro and Sousa 2005). Perhaps a study on the use of testosterone in reindeer to estimate age class should be undertaken in the future.

In our last objective we examined the use of faecal pellet morphometry to identify age structure of a population of reindeer in Svalbard. Overall we found that pellet size could differentiate between age classes and that the most accurate method to estimate age class was found to be the use of a combination of all three dimensions (length, width, and depth). Most successful faecal pellet size studies have been limited to one dimension for age estimation (MacCracken and Van Ballenberghe 1987, Reilly 2002, Southgate 2005, Ball 2010), however we believe that combining these dimensions may improve the accuracy of the method.

Management Recommendations

From our study, we are able to see that non-invasive methods can be extremely useful, however validation of the analysis procedures including assays is always warranted. A combination of faecal pregnane for pregnancy diagnosis, pellet size for age classification (Ball 2010, this study), and DNA techniques for individual identification and sex determination (Huber et al. 2002, Murphy et al. 2003, Harris et al. 2010) could help us determine many important population parameters required to predict future caribou population trends. Faecal DNA capture-mark-recapture techniques are mandatory for individual identification and population size estimates, however the ability to identify the age and reproductive status of the animal will be helpful in estimating future population dynamics. Being able to deduce this information without ever being in contact with the study animal has been our goal all along and therefore, we find that the combination of these methods will aid in the future conservation of many animal species.

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