

**Feasibility of Faecal DNA Genotyping  
as a Noninvasive Population Survey Technique  
for the Canadian Swift Fox (*Vulpes velox*)**

by

Medea M. Curteanu

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree  
of

**MASTER OF NATURAL RESOURCES MANAGEMENT**

Department of Natural Resource Institute

University of Manitoba

Winnipeg, Manitoba

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*I would like to dedicate my thesis to  
the two most important people in my life:  
My mother Rodica, for her everlasting love,  
eminent values, and source of inspiration.  
And to Daniel, for his infinite support, strength, and  
encouragement.  
Thank you!*

## ABSTRACT

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The purpose of this research study was to test the feasibility of estimating swift fox (*Vulpes velox*) numbers by genotyping DNA extracted from faecal samples collected from wild individuals. To determine the optimum season to conduct swift fox population surveys that would provide reliable molecular and population data, two survey methods of collecting fresh faecal samples in both high and low density areas in summer, fall, and winter conditions in southwestern Saskatchewan were compared. Summer and winter samples collected using the bait-station method were first screened using two microsatellite loci and target DNA was quantified. A portion of samples were identified to the species level (mitochondrial DNA) while samples with high quantity and quality target DNA were genotyped to the individual level (microsatellite DNA). As a reliability index, total number of unique genotypes generated from the winter-collected samples was compared with total individuals captured during the live trapping survey.

A total of 478 fresh fox-like faecal samples were collected during the three sampling periods with the highest proportion of samples obtained from the summer roads/trails survey. However, the bait-station method was more species-specific and effective per unit effort, with the highest number of faecal samples obtained during the winter sampling period. Mitochondrial DNA amplification success rate was high (~92%) with no significant difference observed between the two seasons. Conversely, microsatellite amplification of one loci was significantly higher for winter-collected samples (79%) than for summer-collected

samples (52%)( $P < 0.05$ ), while a greater proportion of winter-collected samples yield target DNA quantities in the nanograms range and acceptable for a single step genotyping protocol. The higher number of samples collected combined with a higher proportion of samples producing high quantity target DNA resulted in a greater number of samples being genotyped and more unique genotypes obtained in the winter than in summer sampling period. Based on field and molecular results, winter sampling using the bait-station method for collecting fresh swift fox faecal samples, followed by DNA quantification and sample screening, species identification, and genetic profiling, was found to be a potential method for estimating swift fox numbers in our study area.

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# CHAPTER 1

## Introduction

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### 1.1 THESIS CONTEXT

The swift fox (*Vulpes velox*) is Canada's smallest canid species that inhabits the native mixed- and shortgrass prairies of Saskatchewan and Alberta and is currently listed as "endangered" by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC 2000). Since European settlement, swift fox populations drastically declined and by the mid 1920s the species became eradicated from Canada. Through an intense international reintroduction and captive breeding program, this species has become re-established on the Canadian prairies once again making its introduction one of Canada's most unique conservation success story. The swift fox is an integral part of the rich and diverse shortgrass prairie ecosystem, however, past and current agricultural practices, oil and gas activities, and urban developments place immeasurable pressure on this unique ecosystem making it one of the most endangered biomes in the world (Hoekstra et al. 2005). Furthermore, population threats such as habitat loss, predation, interspecific competition with other canids, accidental kills, and disease outbreaks continue to pose a direct risk to the status of this once extirpated mammal (Pruss et al. in review).

Effective recovery strategies that will achieve delisting the swift fox from the "endangered" species list and maintain viable populations on the Canadian prairie require accurate population distribution and abundance data. In the last

decade, genetic sampling from DNA extracted from noninvasive sources such as hair and faecal material has been developed and tested on a wide range of species. In particular, faecal DNA genotyping offers many advantages particularly for species at risk which are generally rare and inherently difficult to study. The development of alternative non-invasive survey methods has been identified by the World Conservation Union (IUCN) Canid Conservation Group and the Swift Fox Recovery Team as a major priority for the conservation of the swift fox. Pilot projects must however be conducted in order to assess the feasibility of such a technique on the species of interest. For example, field methodologies such as mode and time of sample collection, application to large scale surveys and factors that can improve population estimates must be considered. Additionally, laboratory logistics such as amplification success rates, effectiveness of genetic markers and any technical difficulties associated with the technique must be evaluated. Thus, the goal of this study was to test and optimize both field and laboratory methods in order to determine the feasibility of this approach on estimating swift fox abundance.

## **1.2 PROBLEM STATEMENT**

In accordance with the *Species at Risk Act* (SARA), recovery strategies must be prepared by federal agencies for species listed by COSEWIC as Extirpated, Endangered or Threatened. The current *Recovery Strategy for Swift Fox in Canada* recommended that swift fox population must be surveyed in order

to monitor population trends in abundance and spatial distribution, to evaluate the overall re-introduction success, and to establish if future reintroductions are necessary (Pruss et al. in review). A population census has taken place every five years since the last reintroduction in 1997 (Moehrenschrager & Moehrenschrager 2006). The present method of surveying swift fox populations is through live-trapping which involves capturing live animals in baited traps. This survey technique, in addition to being very labour extensive and expensive, is considered to be invasive and stressful to the animals. The Swift Fox Recovery Team has identified the “replacement of live trapping with less invasive survey techniques” as an objective for the recovery and conservation of the Canadian swift fox (Pruss et al. in review).

Several studies have examined the effectiveness of faecal DNA genotyping as a population survey technique for canid populations but only two have focused specifically on swift/kit foxes (Harrison et al. 2002; Smith et al. 2005). Essentially, these studies were carried out in the United States using different sample collection methods and molecular analysis protocols. Therefore, the application of this novel method has not yet been applied in Canada nor have factors that might influence the success of this technique been explored. Thus, the aim of this study was to examine the feasibility of faecal DNA genotyping as a population survey technique for the swift fox and determine what factors might play a role in the success of obtaining reliable genetic and population data. Accordingly, the development of a faecal-based population survey protocol could potentially become an effective method of estimating and monitoring the

Canadian swift fox population at a large scale, phasing out live trapping, and overall contributing to the scientific knowledge and conservation of this species.

### **1.3 RESEARCH OBJECTIVES**

The aim of this thesis was to investigate the feasibility of faecal DNA as a noninvasive population survey technique for the endangered Canadian swift fox.

The specific objectives of this project were:

1. To develop an effective method of recovering fresh faecal samples from wild swift fox individuals for the purpose of DNA extraction and population estimation;
2. To evaluate mitochondrial and nuclear DNA amplification success and estimate quantity and quality of target DNA extracted from fresh swift fox faecal samples collected in summer and winter conditions;
3. To estimate the number of individual animals captured in a given township based on faecal DNA genotyping and compare to live trapping results; and
4. To provide appropriate recommendations for developing an effective noninvasive survey technique that provides reliable swift fox genetic and population data.



## **1.4 THESIS STRUCTURE**

This thesis was structured in individual chapters to aid with submission for publication. In Chapter 2, I review the current literature on swift fox biology and ecology in both Canada and United States, as well as the current method of surveying the Canadian population. In this chapter I also examine the different approaches used to survey wild populations using faecal DNA genotyping, the challenges presented by this technique, and some solutions that have been proposed in the literature. In Chapter 3, the effect of seasonality, temperature, and exposure on amplification success of mitochondrial and nuclear DNA extracted from fresh faecal samples, as well as quantification of target DNA, was evaluated in order to determine the optimum season and time that sample collection should be carried out. In Chapter 4, I tested two field methods for recovering fresh swift fox faecal samples in both high and low density areas during three seasons: summer, fall, and winter period. Samples collected using the bait-station method were screened and extracts with high quantity target DNA were genotyped to the individual level while a subsample was identified to the species level. I conclude in Chapter 5 with applications of the results of the study and recommendations for developing an effective swift fox population survey using faecal DNA genotyping. Future research steps are also provided in this chapter.

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## CHAPTER 2

### Literature Review

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#### 2.1 BIOLOGY & ECOLOGY OF THE SWIFT FOX

##### 2.1.1 General Description

The swift fox is the smallest member of the canid family (*Canidae*), weighing an average of 2.4 kg and measuring 30 cm in high (Kilgore 1969). The swift fox is easily differentiated from the common red fox (*V. vulpes*) by its smaller body size, relatively large ears, two black patches on each side of the snout, and a black-tipped tail (Banfield 1974; Egoscue 1979). The swift fox is morphologically and ecologically similar to the kit fox (*V. macrotis*) and their taxonomy has been greatly debated. The kit fox, which is native to United States, is found west of the Rocky Mountains while the swift fox is found to the east, and in southern New Mexico, where their ranges overlap, hybridization between the two foxes occurs (Rohwer & Kilgore 1973; Mercure et al. 1993). Based on morphometric and protein electrophoretic comparison, Dragoo et al. (1990) suggested that the two foxes are conspecific and should be regarded as subspecies. However, phylogenetic mitochondrial DNA analyses carried out by Mercure et al. (1993) on a larger sample size found significant genetic difference between the two populations, particularly for the populations separated by the Rocky Mountains, thus they concluded that the two foxes warrant distinct species status.

### 2.1.2 Life History & Behaviour

Swift foxes are nocturnal and secretive, spending the majority of the day sleeping in an underground den and hunting alone during the night (Kilgore 1969; Egoscue 1979; Kitchen et al. 1999). An opportunistic forager, the swift fox feeds on a diversity of prey items including small mammals, birds, insects, carrion, reptiles and plant material, depending on the geographic region, season, and prey availability (Kilgore 1969; Kitchen et al. 1999; Sovada et al. 2001; Harrison 2003). In less temperate areas, mammals compose the major part of the winter diet, whereas insects of the Orthoptera order (grasshoppers, locusts, crickets) are consumed in large volume during the summer period (Kitchen et al. 1999; Sovada et al. 2001). In Canada, jack rabbits (*Lepus townsendii*), ground squirrels (*Spermophilus* spp.), voles, and mice are the major mammal prey species consumed (Carbyn 1998).

Swift foxes reproduce once a year with the onset of the breeding season depending upon latitude and climate (Moehrenschrager 2000). In Canada, breeding season starts mid-February (Moehrenschrager & Macdonald 2003; Smeeton et al. 2003) while in southern United States it can begin as early as December (Kilgore 1969). Gestation period is approximately 51 days (Smeeton et al. 2003) and the pups are born from mid-April to June (Carbyn 1998). Based on counts at natal dens, mean litter size has been estimated to be 3.8 (range 1-8) and this has been found to be correlated to the female's body weight during the breeding season (Moehrenschrager 2000). Both parents provide for the young and the pups emerge from dens in late May to early June, depending on

climatic conditions during the breeding season (Moehrenschlager 2000). Swift fox pups are fully weaned at six weeks (Egoscue 1979; Carbyn 1998) but the young remain in the vicinity of the natal area until September when they are old enough to utilize their parents' entire home range (Kilgore 1969; Andersen et al. 2003). Juveniles are generally designated as adults after one year (Moehrenschlager 2000; Harrison 2003).

Juveniles begin to disperse from the natal area in the fall with peak dispersal occurring in September-October and January-February (Kamler et al. 2004), although the exact time varies with the geographical location. In Canada, similar to the breeding season, juvenile dispersal is delayed. Moehrenschlager (2000) reported a high proportion of juveniles remaining within natal area until January after which they dispersed approximately two home ranges away. Male and female juveniles have similar dispersal distances (app. 15 km) (Moehrenschlager 2000) but significantly more males than females have been reported to disperse (Kamler et al. 2004). Conversely, adult dispersal tends to take place throughout the year and this tends to occur after the death of a mate (Kamler et al. 2004).

Swift foxes are monogamous and mate for life, however polygamy or one helper female at the den site as well as combined litters and den-sharing have been observed (Kilgore 1969; Moehrenschlager 2000; Olson & Lindzey 2002a; Schauster et al. 2002; Kitchen et al. 2005). Kamler al. (2004b) found that monogamous mating occurred in areas of low population density while polygamous groups and non-breeding females occurred in high density areas.

### 2.1.3 Habitat Requirements

The swift fox inhabits the native short and mixed-grass prairie region of North America's Great Plains and prefers open habitats with sparse vegetation and gently rolling terrain that allows for good predator and prey visibility (Kilgore 1969; Carbyn 1998). When different habitat types were compared, swift foxes showed strong selection for grazed short-grass prairies, while agricultural fields were partially used, and irrigated agricultural fields and tall ungrazed non-native grasslands were entirely avoided, even when these habitat types were abundant (Kamler et al. 2003a).

The swift fox is one of the most den-dependent canids spending the majority of the day inside or in the vicinity of the den which is used throughout the year to escape from predators, to give birth, or to rear pups (Egoscue 1979; Kitchen et al. 1999; Kamler et al. 2004). Dens are usually located in elevated areas which are characterized by well-drained sandy loams and clay soils (Kilgore 1969, Harrison et al. 2003, *but see* Tannerfeldt et al. [2003] *for a review on den ecology*). Swift fox dens are commonly found near man-made habitats such as roads, culverts, pipes and buildings, and generally outside coyote (*Canis latrans*) core home-ranges (Kilgore 1969; Pruss 1999; Harrison 2003; Kamler et al. 2003b).

Swift foxes have large home-ranges which vary in size with the geographical location, population density, time of season, or the type of estimator used to calculate this area. Mean annual home-range sizes were reported to be 7.6 - 9.4 km<sup>2</sup> in southern Colorado (Kitchen et al. 1999; Schauster et al. 2002), 11.7 km<sup>2</sup>

in Texas (Kamler et al. 2003a), 14.9 km<sup>2</sup> in New Mexico (Harrison 2003), 15.9 km<sup>2</sup> in Kansas (Sovada et al. 2003), and 18.6 km<sup>2</sup> in Wyoming (Olson & Lindzey 2002b). Home-ranges for Canadian swift foxes are considerably larger (range: 31.8 km<sup>2</sup> to 40.8 km<sup>2</sup>) than those in southern regions and this was suggested to reflect the less abundance of prey species present on the Canadian prairies (Moehrenschrager et al. in press). Although, males and females have similar annual home-range sizes (Moehrenschrager 2000; Kamler et al. 2003a), females were found to have smaller ranges than males during the pup-rearing period when they become den-bound (Olson & Lindzey 2002b). Seasonality can influence the size of the home-range for both adults, with smaller sizes found during pup-rearing period than the dispersal period (Olson & Lindzey 2002b; Kitchen et al. 2005). Swift foxes are territorial (Olson & Lindzey 2002b) although extensive home-range overlap occurs among closely related neighbors (Kitchen et al. 2005).

#### **2.1.4 Population Distribution & History**

Historically, the swift fox was considered an abundant species on the North American prairies and based on pelt records its range occurred east of the Rocky Mountains to western Minnesota and as far south as west-central Texas (Egoscue 1979). In Canada, the swift fox population extended from the Alberta's foothills through southern Saskatchewan east to Manitoba's Pembina Hills (Banfield 1974) and north of the Saskatchewan River (Egoscue 1979). It is suspected that across its entire range the swift fox distribution was never

continuous with some regions always being patchy (Carbyn 1998). During the 1900s, swift fox populations throughout North America dramatically declined as a result of the inadvertent poisoning aimed at eradicating coyotes and wolves (*C. lupus*) in combination with the loss of critical habitat from the conversion of native grassland to farmland (Kilgore 1969; Egoscue 1979; Kitchen et al. 1999). Unregulated trapping and hunting of all fox species, rodent control, widespread use of pesticides and herbicides, and decrease in prey availability have also been attributed to the reduction of swift fox numbers across its range (Carbyn 1998). In Canada, the last swift fox was trapped in 1928 in Govenlock, Saskatchewan (Carbyn 1998) and fifty years after the last sighting, the swift fox was officially designated by the Committee On the Status of Endangered Species (COSEWIC) as “extirpated”, meaning that no individuals existed in the wild in Canada but the species could be found in other parts of its range (COSEWIC 2000). In 1973, two swift fox pairs were imported from Colorado by the Smeeton family of Cochrane, Alberta, who began the first swift fox captive breeding program in Canada (Brechtel et al. 1996; see Herrero 1986 and Moehrensclager & Moehrensclager 2006 for sequence of events). In 1983, the first captive-bred swift foxes were released in Alberta, followed by releases in Saskatchewan a year later (Moehrensclager & Moehrensclager 2001). To ensure maximum genetic diversity within the captive-born population, thus avoiding inbreeding and decreased reproductive success, translocated foxes were obtained from various populations throughout United States (Herrero 1986) and a studbook record has been kept at the breeding facility (Smeeton et al.



2003). From 1983 to 1997, 942 captive-raised and translocated swift foxes were released annually in Alberta and Saskatchewan (Brechtel et al. 1996) and in 1978 the swift fox was down listed to endangered status (COSEWIC 1978). Although, the Canadian swift fox recovery program is considered “the most successful carnivore reintroduction of a nationally extinct carnivore to date” (Moehrenschrager & Moehrenschrager 2006), current population threats pose a direct risk to the status of this species on the Canadian prairies.

### **2.1.5 Mortality & Population Threats**

In general, swift foxes have short life spans (< 5 years) (Harrison 2003) although in captivity they have been reported to live up to 14 years (Smeeton et al. 2003). In the wild, swift foxes coexist with sympatric species like the coyote and the red fox that act as predators or interspecific competitors (Tannerfeldt et al. 2003). Survival rates of swift fox populations vary with the geographical region, age, habitat type, predator density and season. Adult annual mortality rates range from 0.33 to 0.64 whereas juveniles tend to experience a much higher mortality rate (0.87 - 0.95) (Schauster et al. 2002; Tannerfeldt et al. 2003). In Canada, adult and juvenile mortality rates were found to be similar (app. 0.56) but higher rates were reported in the spring than fall period (Moehrenschrager 2000).

Coyote predation has unanimously been reported as the leading cause of mortality in swift fox populations (Kitchen et al. 1999; Olson & Lindzey 2002a; Schauster et al. 2002; Andersen et al. 2003; Harrison 2003; Moehrenschrager et

al. in press) with rates as high as 89% reported in Texas (Kamler et al. 2003b). Predation by coyotes tends to occur on the periphery of swift fox home-range as a result of unfamiliarity and inability of foxes to locate escape holes (Kitchen et al. 1999; Moehrenschrager 2000; Olson & Lindzey 2002a; Moehrenschrager et al. in press). In areas where coyote populations were controlled by local landowners and/or recreational hunting, swift fox density, recruitment, and survival rates increased (Kamler et al. 2003c).

Other causes of swift fox mortality include raptor and badger (*Taxidea taxus*) predation, canine distemper, shooting, poisoning, incidental trapping, starvation, and vehicle collisions (Carbyn 1998; Moehrenschrager 2000; Olson & Lindzey 2002a; Kamler et al. 2003a). Since swift fox dens are often located in close proximity to roads (Pruss 1999) or in high road density areas (Kintigh & Andersen 2005), both adults and juveniles are frequently killed by vehicles (Kamler et al. 2003a; Tannerfeldt et al. 2003).

In Canada, the primary threats to swift foxes are habitat loss, habitat fragmentation, predation by coyotes, and interspecific competition with red foxes (Carbyn 1998; Moehrenschrager 2000; Moehrenschrager & Sovada 2004; Pruss et al. in review). Swift foxes are prairie specialists, thus availability of large tracks of native short-grass prairie is essential for their survival. Ongoing habitat loss, fragmentation, and degradation through the conversion of native grassland to agricultural use, together with the increase of oil and gas activities across the prairies, is decreasing suitable habitat and limiting swift fox distribution (Moehrenschrager 2000). Direct predation by coyotes in combination with

resource competition through dietary overlap and invasion of dens by red foxes are negatively impacting swift fox recovery (Moehrenschrager 2000; Moehrenschrager & Sovada 2004). The possibility of a disease outbreak is also an underlying population threat particularly since canine distemper and parvovirus have been reported to be prominent in high swift fox density areas (Moehrenschrager & Moehrenschrager 2006). Other factors such as access to adequate den sites has been suggested to restrict swift fox distribution (Tannerfeldt et al. 2003; Kintigh & Andersen 2005) while winter prey availability has been speculated to limit swift fox survival and population growth on the Canadian prairies (Klausz et al. 1996).

#### **2.1.6 Monitoring Strategies**

In 1989, a National Swift Fox Recovery Team was established with the primary goal “to increase populations of the swift fox so that, by the year 2000, they are self-sustaining and stable or increasing, the species is no longer considered endangered in Canada, and biotic diversity on the prairies is enhanced” (Brechtel et al. 1996; COSEWIC 2000). Since the last re-introduction in 1997 a nation wide swift fox census has been conducted every 5 years to assess the distribution and viability of the reintroduced population (Cotterill 1997; Moehrenschrager & Moehrenschrager 2001; Moehrenschrager & Moehrenschrager 2006) and determine if future re-introductions are necessary. The first population survey revealed that although the reintroduced foxes were becoming established and breeding successfully in the wild, their distribution was

fragmented, consisting of two isolated subpopulations, one spanning the borders of Alberta-Saskatchewan, and the second in and around Grassland National Park Region of south-central Saskatchewan (Cotterill 1997). However, the recent 2005-2006 population census showed positive results. During this survey, 104 foxes were caught, marked, and released in Canada with an additional 92 foxes captured in northern Montana; 100% of these animals were born in the wild (Moehrenschlager & Moehrenschlager 2006). Using the same estimate technique applied in previous censuses (*see Current Survey Methods section below*), the current Canadian population was estimated to be around 647 individuals (Moehrenschlager & Moehrenschlager 2006). In comparison to the 2000-2001 census, the Canadian population has not changed in abundance indicating that the population is stable (Moehrenschlager & Moehrenschlager 2006). Overall, the re-introduced population has significantly increased in size and distribution since the 1997 census, expanding into areas previously uninhabited and connecting the two previously isolated Canadian populations through a traveling corridor in northern Montana (Moehrenschlager & Moehrenschlager 2006).

Eighty years after their eradication and twenty four years after the first introduction, the Canadian swift fox population seems to be established and successfully breeding in the wild, however, the population is still small and regarded “endangered” under the Canadian *Species at Risk Act* (SARA). Since the Canadian-Montana population is considered isolated from the larger United States population (Moehrenschlager & Sovada 2004), stochastic events such as

disease outbreaks, changes in predator dynamics, or severe winter conditions, combined with unremitting anthropogenic changes to the prairie landscape, are threatening the status of the Canadian swift fox population. Given the current population pressures, the 2007 Swift Fox Recovery Team recommended the continuation of monitoring and conservation programs and that “by 2026, restore a self-sustaining swift fox population of 1000 or more mature, reproducing foxes that does not experience greater than a 30% population reduction in any 10-year period” and (Pruss et al. in review). Thus, ongoing studies to determine abundance, geographic distribution, density, habitat selection, and population trends over the next years are fundamental to the successful recovery and sustainability of the swift fox on the Canadian prairies.

## **2.2 POPULATION ABUNDANCE & SAMPLING METHODS**

### **2.2.1 Current Survey Methods**

Absolute abundance, or the actual number of individuals in a population (Harrison et al. 2002; Lettink & Armstrong 2003), is an important parameter for monitoring and developing effective recovery plans for the endangered swift fox. The current method to estimate population abundance and geographic distribution is by the conventional live-trapping method where animals are captured in baited cages and an identification tattoo is given to each animal before being released back in the wild (Moehrensclager & Moehrensclager 2001, 2006). Animals are re-caught over a three night period and a correction

factor developed by Cotterill (1997) is used to estimate absolute abundance. The survey is conducted during the winter months in order to prevent capturing and stressing the animals during the breeding, gestation and pup-rearing period (Cotterill 1997; Moehrenschlager & Moehrenschlager 2006). There are many advantages of live-trapping including the fact that swift foxes readily enter baited cages and an accurate species and individual identification is obtained from marked individuals (Harrison et al. 2002). The collected data can be used to extrapolate valuable population parameters such as distribution, density, sex ratio, age dynamics, and overall health of the population (Moehrenschlager & Moehrenschlager 2006). For reintroduced species like the Canadian swift fox, live-trapping has provided valuable data on number of wild-born individuals versus captive-raised or translocated foxes (Moehrenschlager & Moehrenschlager 2001). Furthermore, since swift foxes are susceptible to diseases and parasites (Brechtel et al. 1996), live-trapping provides the opportunity to collect blood and parasite samples for laboratory analysis (Moehrenschlager & Moehrenschlager 2001; Harrison 2003).

Several problems exist with the live-trapping method. In addition to posing risk of injury to individuals that are already rare and endangered (Kohn et al. 1999; Harrison et al. 2002; Smith et al. 2005), data obtained from this method can be biased meaning that the probability of capturing animals is not equal across all individuals in the surveyed population. The probability of trapping an animal depends on numerous factors including its behaviour (ie. finding and entering a trap or station) (Harrison et al. 2002; Lettink & Armstrong 2003),

weather conditions during sampling period, or changes in survey effort (Burnham & Overton 1979). Furthermore, live-trapping is extremely time consuming and labor intensive requiring several months of systematic surveys over large areas and rough terrain (Moehrensclager & Moehrensclager 2001; Wilson et al. 2003).

### **2.2.2 Noninvasive Genetic Sampling**

The term “noninvasive sampling” refers to the method used to collect genetic information (ie. DNA) from sources such as hair or faecal material (scat) without capturing, handling or disturbing the animals (Taberlet et al. 1999; Pompanon et al. 2005). Over the last decade, DNA profiling from faecal material has been applied to answer a wide range of research questions including population abundance, geographic distribution, genetic diversity, phylogeny, hybridization, kinship, sex ratio, movement, and home range size (Paxinos et al. 1997; Kohn et al. 1999; Ernest et al. 2000; Adams et al. 2003; Creel et al. 2003; Piggott & Taylor 2003b; Wilson et al. 2003) and examine population dynamics over a long-term period (Prugh et al. 2005). For species that are difficult to study because they are nocturnal, elusive, endangered, or have large home ranges, noninvasive sampling is a powerful research and management tool (Paxinos et al. 1997; Kohn et al. 1999; Adams et al. 2003; Piggott & Taylor 2003b; Romain-Bondi et al. 2004; Wasser et al. 2004; Prugh et al. 2005). Faecal material offers numerous advantages over live-trapping in the ability to acquire a large sample

size, surveying over large areas, and with possibly less biased data since all animals defecate regularly (Taberlet & Luikart 1999; Fernando et al. 2003; Smith et al. 2005).

Faecal DNA has become invaluable in facilitating positive species identification among closely related species (Mercure et al. 1993; Paxinos et al. 1997; Harrison et al. 2002; Lucchini et al. 2002; Prugh et al. 2005; McKelvey et al. 2006; Sugimoto et al. 2006) and between hybrids (Adams & Waits 2007). Ernest et al. (2000) found that DNA obtained from mountain lion faecal material can be used to reliably differentiate from closely related sympatric species such as cougars and feral cats as well as from consumed prey species that were present in the faeces. For the purpose of individual identification and population studies, faecal DNA has been successfully carried out for a wide range of species including coyote (Kohn et al. 1999; Prugh et al. 2005), wolf (*Canis lupus*) (Lucchini et al. 2002; Adams et al. 2003; Creel et al. 2003), forest elephant (*Loxodonta cyclotis*) (Eggert et al. 2003), brown bear (*Ursus arctos*) (Bellemain et al. 2005), black bear (Wasser et al. 1997; Bellemain et al. 2005), European badger (*Meles meles*) (Wilson et al. 2003) and fox species (Harrison et al. 2002; Smith et al. 2005). Greater genotyping success from faecal than hair DNA has been reported from several species including Canada lynx (*Lynx Canadensis*) (McKelvey et al. 2006) and bobcat (*Lynx rufus*) (Ruell & Crooks 2007). To verify the accuracy of this technique, several studies have compared tissue and/or blood samples with faecal DNA obtained from same individuals and these produced identical genotypes (Wasser et al. 1997; Kohn et al. 1999).



Additionally, canid-specific primers for sexing individuals from faecal DNA have been developed and found to be 90-100% accurate (Ortega et al. 2004; Prugh et al. 2005; Seddon 2005).

### **2.2.3 Estimating Population Abundance**

Similar to live-trapping methods, population abundance via noninvasive sampling can be estimated using capture-recapture models or directly counting the number of individuals, the difference being that unique genotypes rather than live-marked animals are recorded (Lettink & Armstrong 2003; Lukacs & Burnham 2005b; Prugh et al. 2005). Capture-recapture models using DNA obtained from trapped hair have been applied extensively in estimating grizzly bear (*Ursus arctos*) and black bear (*Ursus americanus*) abundance in British Columbia and Alberta (Woods et al. 1999; Romain-Bondi et al. 2004; Wasser et al. 2004). Mark-recapture models can be applied to closed or open populations (Lettink & Armstrong 2003). Most faecal genotyping studies have used closed-population models using the Lincoln-Petersen estimator which assumes a constant population during sampling period (e.g. Kohn et al. 1999, Harrison et al. 2002, Frantz et al. 2003, Wasser 2004). Although more difficult because population parameters such as births, deaths, immigration or emigration must be taken into account (Lettink & Armstrong 2003), faecal mark-recapture sampling for open populations has been carried out. Prugh et al. (2005) evaluated this approach by sampling a wolf population in Alaska over a 3 year period and concluded that

faecal genotyping is an effective approach for monitoring populations over a long time scale.

Another method of estimating absolute abundance is to conduct a systematic search where all individuals are “captured” or detected. Initially, the genotype recovery rate is exponential as every sample yields a new individual but eventually it levels off as more of the population is identified or captured (Kohn et al. 1999). When compared to live-trapping population data, both Kohn et al. (1999) and Wilson et al. (2003) reported that the rarefaction analysis using microsatellite genotyping produced similar population abundance estimates.

## **2.3 FAECAL DNA GENOTYPING**

### **2.3.1 Introduction**

Faecal material contains discarded epithelial cells that lined the intestinal walls of the individual (Wasser et al. 1997; Maudet et al. 2004). It is the DNA within these cells that is extracted and amplified through multiple polymerase chain reactions (PCRs), the enzymatic process where small amounts of target template DNA are made into high copy number for individual characterization (ie. genotyping) (Taberlet et al. 1996; Nsubuga et al. 2004). In most organisms, DNA is found in the nucleus and cellular organelles. In animals, organelle DNA is found only in the mitochondria while in plants it is found in the chloroplast (Krawczak & Schmidtke 1994). The mitochondrial DNA (mtDNA) is circular and small, about 15 to 20 thousand base pairs (bp) in length depending on the species (Parker et al. 1998). A single mammalian cell has several thousand

mitochondria, making up to 0.5% of total cellular DNA (Krawczak & Schmidtke 1994). Mitochondrial DNA is maternally transmitted and does not undergo recombination (Parker et al. 1998). In comparison to nuclear DNA, mtDNA regions have a much higher mutation rate (6 to 17 times), (Krawczak & Schmidtke 1994), thus it is widely used to differentiate among closely related species and in the detection of population structure (Hartl & Clark 1997).

Nuclear DNA is much larger than mtDNA, ranging from 4.7 million base pairs in some bacteria up to 230 million base pairs in the largest human chromosome (Hartl & Clark 1997). Microsatellites, which are repetitive short tandem noncoding units of 2-8 nucleotides in length, are dispersed throughout the entire nuclear genome (Krawczak & Schmidtke 1994). These sequences are highly variable and their variation is extremely useful in generating multi-locus genotypes which can be used to identify individuals (Pompanon et al. 2005). Due to the high copy number (~ 1000 copies per cell), mtDNA is easy to analyze (Krawczak & Schmidtke 1994). Conversely, nuclear DNA can be found in limited amounts (2 copies of each gene per cell), thus is much more problematic to analyze particularly when extracted from faecal material. This is because nuclear loci extracted from faecal samples can only yield a few picograms (pg) of target DNA required for genotyping (Gerloff et al. 1995; Taberlet et al. 1996; Pompanon et al. 2005), while the quality can be poor as a result of degradation and presence of dietary inhibitors (Wasser et al. 1997). Damage or fragmentation of nuclear DNA occurs when samples are exposed to environmental elements such as high temperature, moisture, and ultraviolet radiation (Wasser et al. 1997;

McKelvey & Schwartz 2004a). Inhibitors, such as bilirubin and bile salts, occur from digested food sources and these are known to copurify both mtDNA and nuclear template DNA overall hindering amplification success if not removed during the isolation process (Kreader 1996; Prugh et al. 2005). The presence of PCR inhibitors can be minimized by using a silica-based commercially purification kit, in combination with an ethanol wash (Wasser et al. 1997; Ye et al. 2004; Ball et al. 2007).

### **2.3.2 Amplification Success**

Faecal-based studies have shown great variance in DNA quality and amplification success (Scandura et al. 2006; Broquet et al. 2007). An array of confounding factors including sampling season (Lucchini et al. 2002; Maudet et al. 2004), sample age (Piggott 2004), preservation techniques (Murphy et al. 2002; Roeder et al. 2004), DNA extraction methods (Piggott & Taylor 2003a), environmental conditions (Nsubuga et al. 2004), as well as the species of interest and its diet (Murphy et al. 2003), have all been found to affect the overall amplification success rate. For example, faecal DNA amplification rate has been shown to correlate with the age or freshness of the sample as well as with the collection season (Lucchini et al. 2002; Piggott 2004). Both Lucchini et al. (2002) and Maudet et al. (2004) reported higher success rates for winter-collected samples than for summer or spring samples. Prugh et al. (2005), who carried out their survey in winter conditions, reported that age of samples, which varied from

1 to 90 days, did not affect amplification success. The higher amplification rates recorded in winter-collected samples is believed to be influenced by the low temperatures that reduce the DNA degradation rate by endo- and exonuclease enzymes (Nsubuga et al. 2004; Prugh et al. 2005). Furthermore, the species' food habits during sampling season have been suggested to play a significant role in DNA amplification success rate (Maudet et al. 2004).

On a technical level, the protocol used for sample preservation and DNA extraction has been shown to improve DNA amplification success rate (Wasser et al. 1997; Murphy et al. 2002; Nsubuga et al. 2004; Roeder et al. 2004; Wehausen et al. 2004). For the Eurasian badger a higher amplification rate was achieved from samples preserved in 70% ethanol than from samples stored in a buffer solution or frozen (Wilson et al. 2003), while coyote faecal samples stored at -80C in buffer solution had higher DNA amplification success rate than samples stored at -80C without buffer (Prugh et al. 2005). Wehausen et al. (2004) reported higher amplification rates for extracts where scrapings from the outer layer of sheep faecal pellets were used than for extracts where the inner material was included. This protocol assumes that the outer layer of the faecal sample contains a significant amount of high quality DNA originating from the intestinal tract of the target species and contamination from PCR inhibitors and foreign DNA found throughout the faecal sample are minimized (Flagstad et al. 1999; Fernando et al. 2003; Maudet et al. 2004; Ball et al. 2007). In Australia, the most effective technique for preserving and extracting DNA from red fox faecal material was air-dried followed by a surface wash and spin column purification

(Piggott and Taylor 2003). No standard preservation or extraction method has been established for the swift fox.

### **2.3.3 Genotyping Error**

Successful PCR amplification of target DNA is not an indication that reliable results have been obtained. Because faecal DNA is typically of low quantity and/or fragmented (ie. degraded), genotyping errors generally occur (Waits & Leberg 2000; Creel et al. 2003). Genotyping errors arise when the established genotype does not correspond to the real genotype of the individual in the studied population (Taberlet et al. 1996; Pompanon et al. 2005). Two types of genotyping errors have been identified. The first, referred to as the “shadow effect”, occurs when the genotypes of different individuals are claimed to originate from a single individual thus resulting in fewer individuals being identified and overall underestimating the true population (Mills et al. 2000). This type of error can be generally avoided by using sufficient number of loci (ie. 7 or greater) that are highly variable and independent, thus able to differentiate among unique individuals (Mills et al. 2000). The second type of error, known as either “allelic dropout” or “false alleles”, occurs when multiple genotypes from different samples are created or recorded for the same individual causing excess individuals to be identified and overall overestimating the true population size (Taberlet et al. 1996; McKelvey & Schwartz 2004b). Allelic dropout occurs when one allele of a heterozygous locus fails to amplify resulting in a homozygote

locus; false alleles are allele-like artefacts that occur during the PCR process when a heterozygous at a homozygous locus or three alleles at a heterozygous locus are created (Pompanon et al. 2005). The occurrence of false alleles is less common and easier to identify from the unusual pattern of the alleles, therefore the samples can be reanalyzed and the genotyped correctly (Taberlet et al. 1996). In contrast, allelic dropout occurs more frequently than false alleles (Lucchini et al. 2002) and their occurrence can go unnoticed. Allelic dropout has been reported even in high quality samples such as blood, yet their exact cause is not fully understood (Soulsbury et al. 2007).

Microsatellite genotyping errors are a serious problem for population genetic studies particularly since an error at a single locus can create a false individual such that when multilocus genotypes are produced for individual identification, population size can be overestimated by as much as 200% (Waits & Leberg 2000; Creel et al. 2003). Harrison et al. (2002), who carried out the only swift fox faecal DNA-based study in New Mexico, reported that sampling method overestimated the population more than 5 times as a result of genotyping errors. For capture-recapture population models, an important assumption is that individual tags are read correctly and are not lost during sampling periods (Lettink & Armstrong 2003; Lukacs & Burnham 2005a). Thus, population estimates are extremely sensitive to genotyping errors and the creation or loss of tags can drastically affect recapture rates and overall bias the results of the study (McKelvey & Schwartz 2004b; Roon et al. 2005). The significance of genotyping errors on biological data has received significant attention in recent years,

particularly when estimating population abundance for species at risk or using capture-recapture models (Creel et al. 2003; Bonin et al. 2004; McKelvey & Schwartz 2004a; Lukacs & Burnham 2005b; Pompanon et al. 2005; Roon et al. 2005). Numerous protocols have been suggested to prevent, track, and account for genotyping errors in data sets associated with population estimation and several of these approaches are addressed below.

**Multiple-tubes approach:** The multiple-tubes approach developed by Taberlet et al. (1996) has been adopted in the field of noninvasive genetic sampling as the accepted method to reduce error rates and obtain reliable genotypes (eg. Piggott & Taylor 2003 a; Eggert et al 2003; Wilson et al. 2003). The multiple-tubes protocol accounts for stochastic pipetting, possible allelic dropout and false alleles, and sample contamination, and is based on two rules. First, an allele has to be observed at least twice before it is accepted and secondly, a homozygous locus is not accepted until it has been confirmed 7 times (Taberlet et al. 1996). The principle behind this approach is that the occurrence of incorrect genotypes will decrease by increasing the number of amplifications (Taberlet et al. 1996). Since several PCRs are performed for each locus, this approach becomes costly and time consuming particularly when multilocus genotypes are required (Ball et al. 2007). Furthermore, there is high risk that the template DNA will be consumed before results are obtained (Adams & Waits 2007). It is important to note that this conservative approach was recommended for samples where the amount of template DNA is very low or when the quantity of DNA is not known (Taberlet et al. 1996).



**Quantification of target DNA:** More recently, laboratory methods have been developed to quantify or characterize the amount of total nuclear and target DNA prior to genotyping and remove poor quality samples from the data set (Morin et al. 200, Ball et al. 2007). The logic of this screening approach is that some samples will have high quantity and quality target DNA where multiple PCRs would be unnecessary and costly. For example, Ball et al. (2007) estimated target DNA for winter collected caribou pellets to be over 1000 nanograms illustrating that these estimates are well above the previously reported amounts of few picograms that require the multiple-tubes approach. Thus, quantifying the amount of target DNA can eliminate PCR redundancy and prevent error rates while poor quality samples can be either discarded or reanalyzed.

**Incorporate genotyping errors in data set:** Because allelic dropout has been reported from studies utilizing high quality DNA from sources such as blood and tissue (Hoffman & Amos 2005; Soulsbury et al. 2007), some authors have argued that genotyping errors might not be completely removed from the data set thus other means to deal with them must be established (Lukacs & Burnham 2005b). Creel et al. (2003) suggested a “matching approach” where samples with mismatches at one or two loci be recorded as originating from the same individual thus reducing the risk of overestimation.

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## **CHAPTER 3**

### **Influence of Seasonality and Temperature on Quantity and Quality of Swift Fox Faecal DNA**

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#### **3.1 INTRODUCTION**

Population parameters derived from noninvasive genetic surveys are becoming widespread in conservation genetics and wildlife management particularly for species that are endangered, elusive, or exist at low densities over vast areas (Kohn et al. 1999; Adams et al. 2003; Eggert et al. 2003; Wilson et al. 2003; Bellemain et al. 2005; Prugh et al. 2005; Smith et al. 2005). For species at risk such as the swift fox (*Vulpes velox*), faecal DNA genotyping offers numerous advantages over the traditional live-trapping survey technique, including the ability to sample populations without handling or disturbing the animals, acquiring a large sample size, surveying over large areas, simpler collection and storage methods, and possibly less biased data since all animals defecate regularly (Taberlet & Luikart 1999; Fernando et al. 2003; Smith et al. 2005). However, faecal-based studies have shown great variance in amplification success rate as a result of various factors including sampling season (Lucchini et al. 2002; Maudet et al. 2004), sample age (Piggott 2004), preservation techniques (Murphy et al. 2002; Roeder et al. 2004), DNA extraction methods (Piggott & Taylor 2003), environmental conditions (Nsubuga et al. 2004), and the species of interest and its diet (Murphy et al. 2003). Consequently, these confounding factors affect the quantity of target DNA (the specific template DNA

that enters into PCR) and the quality (ie. level of degradation and/or DNA-to-inhibitor ratio) (Ball et al. 2007). This is because, target DNA may be limited to only a few picograms (pg) in some samples (Gerloff et al. 1995; Taberlet et al. 1996; Pompanon et al. 2005) and coupled with the presence of foreign DNA from consumed prey species (Fernando et al. 2003; Broquet et al. 2007), enzyme activity (Nsubuga et al. 2004; Prugh et al. 2005), and PCR inhibitors such as bilirubin and bile salts found in food sources (Kreader 1996; Prugh et al. 2005), faecal DNA runs the risk of degradation and high genotyping error rates. Genotyping errors (allelic dropout and/or false alleles) are routinely reported in faecal-based studies as a major drawback in obtaining reliable population data (Gerloff et al. 1995; Taberlet et al. 1996; Taberlet et al. 1999; Morin et al. 2001; Pompanon et al. 2005). Genotyping errors can have serious implications because they seem to create new genotypes or non-existent individuals in the true population which can lead to overestimating population size by as much as 200% (Waits & Leberg 2000). Accurate population estimates are especially relevant to mark-recapture studies and population estimates of endangered or at risk species for which error-prone data can lead to poor management decisions and overall negatively impact the species' recovery.

Although, faecal DNA genotyping has the potential to become a successful population survey technique for the Canadian swift fox, effective sample collection methods and lab protocols that minimize genotyping errors have not yet been developed for this species. Currently, it is unclear in which season swift fox sample collection should be carried out such that target DNA

would have high amplification success and low genotyping error rate. For example, in Australia, Piggott et al. (2004) reported higher amplification rates for red fox (*V. vulpes*) summer-collected samples than for spring-collected samples, while Lucchini et al. (2002) reported higher DNA quality for wolf (*Canis lupus*) faecal samples collected on snow than for summer samples. To date, all fox faecal-based studies have been carried out in temperate zones where temperatures generally do not fall below freezing (Harrison et al. 2002; Piggott 2004; Smith et al. 2005), thus winter amplification rates have not yet been reported for foxes. Furthermore, target DNA extracted from canid faecal samples collected in different seasons has not yet been quantified, nor possible factors that affect amplification success have been identified. Thus, evaluating the quantity and quality of target DNA and possible environmental factors that minimizes genotyping errors (ie. allelic dropout) could have significant benefits for the development of a successful swift fox survey protocol.

Fluorescence, the absorbance of light at one wavelength and emission at a different wavelength, has become a widespread technique in molecular biology since it is highly sensitive, inexpensive, and readily accessible to purchase (Ahn et al. 1996; Tolun & Myers 2003; Ball et al. 2007). In particular, PicoGreen<sup>TM</sup> fluorescence assays have been used to quantify total and target DNA extracted from woodland caribou faecal samples for screening purposes prior to genotyping (Ball et al. 2007). The quantification method is based on the ability of PicoGreen<sup>TM</sup> dye to selectively bind and intersperse between the stacked base pairs of double-stranded DNA and emit fluorescence when excited by laser (Ahn

et al. 1996). The level of fluorescence, which is detected by a fluorometer, is proportional to the amount of bound PicoGreen<sup>TM</sup> and thus total double-stranded DNA (dsDNA) in the sample. However, PicoGreen<sup>TM</sup> estimates are not an exact representation of total nuclear DNA of the target species but rather a measure of all genomic DNA in the sample which in the case of faecal samples could originate from other non-target sources such as: i) animals or plants consumed by the target species, or iii) bacteria and/or parasites present in the feces (Bradley & Vigilant 2002; Ball et al. 2007). Thus, the amount of species target DNA can be estimated by measuring total nuclear DNA using PicoGreen<sup>TM</sup> assays, diluting samples at desired concentrations, and comparing amplified sample products with species DNA at known concentrations (see Ball et al. 2007 for details). As such, samples that provide 0.5-5.0 ng per reaction of target DNA or greater could be considered in the range that minimizes allelic drop-out and could be used for simple genotyping protocols (Ball et al. 2007). For example, quantification of caribou faecal samples reported a risk of allelic dropout at less than 0.2 ng (Ball et al. 2007), while human forensic analysis, from which noninvasive sampling stems from, utilizes 0.5-2.0 ng of target DNA (Leclair et al. 2003). In addition to estimating target DNA quantity, the quality of template DNA can also be evaluated by examining the ratio between total estimated nuclear DNA and the amplified sample target DNA (Ball et al. 2007). A 1:1 ratio of total to target DNA would indicate that the PicoGreen<sup>TM</sup> estimate was correct and that the sample contains high quality target DNA from the target species, while a 1:2 ratio would indicate that the PicoGreen<sup>TM</sup> estimate represents only half of the

target species' DNA thus some level of foreign DNA possibly from bacteria or consumed prey species is present in the sample.

The objective of this study was to examine the impact of two seasons (summer and winter) on the amplification success of mtDNA and nuclear DNA extracted from fresh swift fox faecal samples in order to determine the optimum sampling season for conducting non-invasive surveys. Additionally, using fluorescent assays and adopting the two-step characterization protocol developed by Ball et al. (2007), swift fox target DNA was quantified, while environmental parameters that might play a significant role, such as temperature and exposure, were evaluated. Thus, by examining mtDNA and nuclear DNA amplification success and quantifying target DNA from these two seasons, the results of this study can determine the optimum season for carrying out swift fox faecal collection and overall aid in the development of an effective noninvasive population survey protocol for this endangered species.

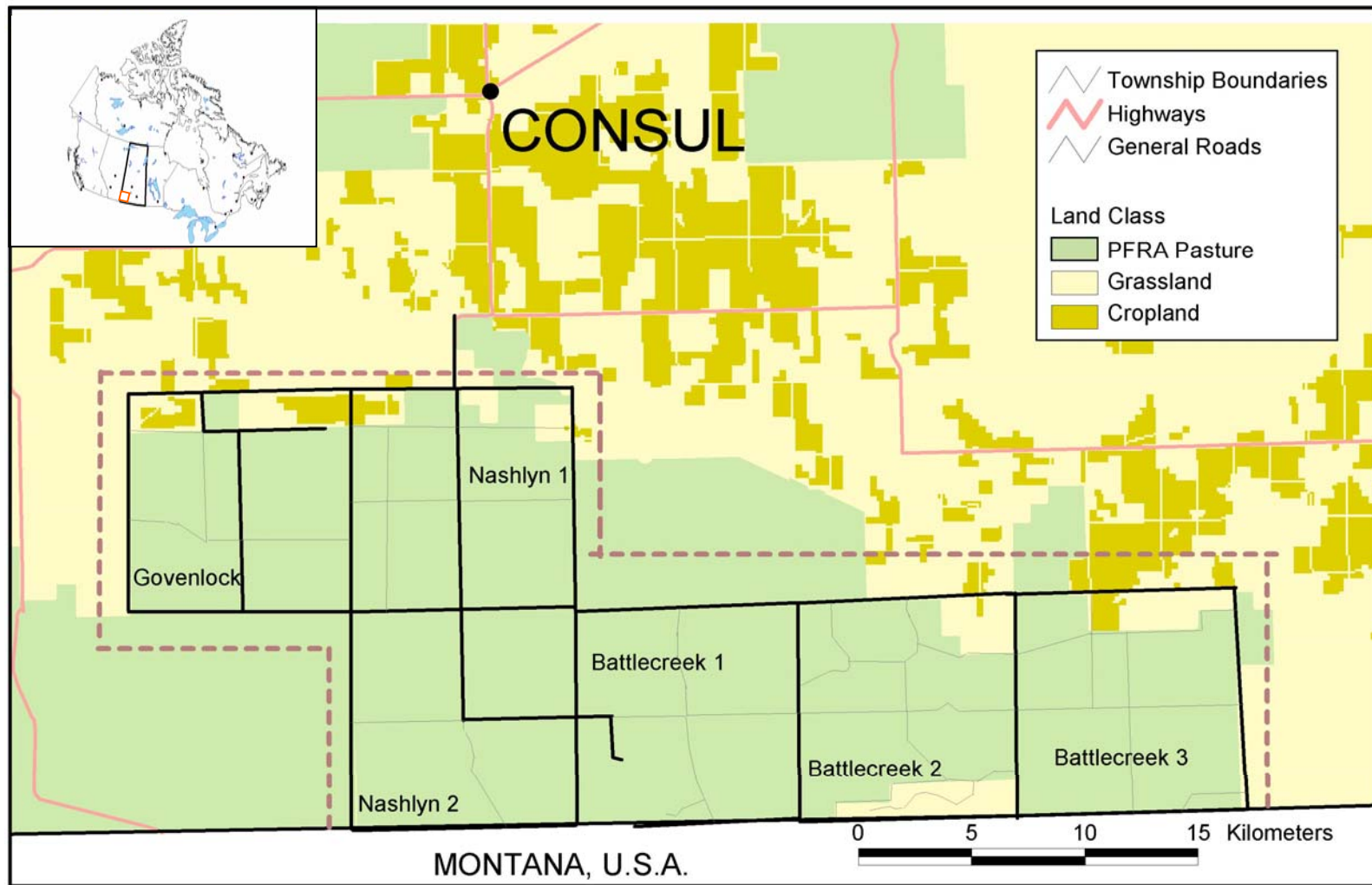
## **3.2 METHODS**

### **3.2.1 Study area**

The study was conducted in southwestern Saskatchewan, north of the Montana border and east of the Alberta border, on federal community pastures managed by the Prairie Farm Rehabilitation Administration (PFRA) (Figure 1). The study site, spanning an area of 565 km<sup>2</sup>, consisted of six townships which were exclusively utilized for cattle grazing. Regional climate is semi-arid

characterized by hot, dry summers and cool, dry winters where temperature can reach as high as 40°C and as low as - 43°C (Environment Canada 2005). The predominant ecosystem is the native shortgrass prairie which is composed of the *Stipa-Bouteloua-Agropyron* vegetation type characteristic of the region's shallow sandy loam soils (Moehrenschrager 2000). The dominant grass species are needle and thread (*Stipa comata*), blue grama (*Boutela gracilis*), northern wheatgrass (*Agropyron dasystachyum*) and western wheat grass (*Agropyron smithii*) but forbs and shrub species such as moss phlox (*Phlox hoodii*), broomweed (*Gutierrezia sarothrae*), pasture sage (*Artemisia frigida*), winterfat (*Eurotia lanata*), salt sage (*Artemisia tridentata*) and sagebrush (*A. cana*) are also common (Moehrenschrager 2000). Elevation ranged from 850 to 1050 m above sea level (Moehrenschrager 2000).





**Figure 1** - Map of study areas, Saskatchewan, 2005 -2006. Townships where surveys were carried out are outlined in the dotted red line (Source data taken from PFRA 1:50000 map).

### **3.2.2 Sample Collection**

Swift fox faecal samples were collected in late summer (August-September) and early winter (November-December) of 2005 from bait-stations established within the center of each township at 0.5-km intervals along a 5-km transect (Sargeant et al. 2003; Uresk et al. 2003). A bait-station consisted of a wooden stake wedged in the ground and baited with approximately 20 g canned sardines packed in oil (Uresk et al. 2003) which was placed on top of the stake. Because fresh faecal samples have been shown to have higher amplification success and lower genotyping error rates than older samples (Lucchini et al. 2002; Piggott 2004), bait-stations were revisited each day for four consecutive days in order to ensure that samples were fresh. Samples found near the bait-station were collected in sterile labeled plastic bags and placed in a small cooler containing two frozen ice packs. To avoid cross-sample contamination, a new stick was used to collect each sample, and scats located further than 5 cm apart were treated as separate samples. The location of each sample was geo-referenced and specific data, such as collection time, scat freshness and age, were entered in a Personal Data Assistant (PDA) (Trimble GeoXM) using the CyberTracker software. The age of majority of samples was less than 1-day old, with the exception of five winter-collected samples which were less than 2-day old. At the end of each day samples were transferred to a freezer and stored at –20°C for 9-12 months. Samples were transported frozen to the Natural Resources DNA Forensics and Profiling Centre (NRDFPC) at Trent University, Ontario for genetic analysis.

### **3.2.3 DNA Extraction**

DNA was extracted from a total of 139 fox-like faecal samples (63 summer and 76 winter). Samples were thawed at room temperature for several minutes and re-hydrated with 3 ml of 0.1M Phosphate Buffered Saline (PBS) solution for several minutes. The outer surface of each sample was gently swabbed with a sterile cotton applicator targeting the outer mucous layer of the scat (Ball et al. 2007). Once the whole sample was swabbed, the applicator was immersed in 250 µL 1X ASL lysis buffer (4 M urea, 0.2 M sodium chloride, 0.5% n-lauroyl sarcosine, 10 mM CDTA (1, 2-cyclohexanediaminetetraacetic acid), 100 mM Tris-HCL pH 8.0) (Applied Biosystems), and vigorously stirred. Digestion of samples was carried out using 20 units of Proteinase K enzyme (Qiagen Inc., Mississauga Ontario) followed by 30 seconds vortex, and incubation in a 70°C water bath for 2 hours. An additional 20 units of Proteinase K was added and allowed to incubate at 37°C overnight. DNA was extracted using the QiaAmp DNAeasy extraction kit (Qiagen Inc., Mississauga Ontario) following the protocol for extraction of mammal tissue. The resulting solution which served as stock for subsequent assays was stored at below zero temperature in a DNA-designated fridge.

Several measures were taken throughout the study to minimize potential contamination: 1) individual filtered pipette tips with aerosol filters were used for each sample; 2) amplification mixture was prepared in a non-DNA area using non-DNA equipment; 3) negative controls, samples without DNA, were included in each reaction, and 4) PCR cocktail was prepared in a non-DNA area to prevent contamination from pre-amplified DNA.

### 3.2.4 Species Verification

To determine if isolated DNA originated from other sympatric mammals such as red foxes, coyotes (*Canis latrans*), or badgers (*Taxidea taxus*), species verification was carried out on 77 (30 summer and 47 winter) randomly selected samples by amplifying a portion of the mtDNA control region using the forward primer – L15997 and reverse primer – H16498 (Kocher et al. 1989). Cycling conditions for PCR followed 94°C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s followed by final extension at 72 °C for 2 min. Confirmation of product was visualized on a 1.5% agarose gel stained with ethidium bromide. PCR products were purified for sequencing using ExoSAP-IT (USB) following the manufacturers' instructions. Sequencing was carried out using DYEnamic™ ET terminator cycle sequencing kit, and the resulting fragments were analyzed on a MegaBACE 1000 (GE Healthcare). Fragments were visually inspected, corrected and aligned manually in BioEdit (Hall 1999). Species confirmation and haplotypes were determined for each sample by performing BLAST searches between established sequences and those published in the GenBank public database ([www.ncbi.nlm.nih.gov/Genbank](http://www.ncbi.nlm.nih.gov/Genbank)).

### 3.2.5 Total DNA Quantification

Total amount of nuclear DNA present in each sample was estimated by PicoGreen™ (Molecular Probes) assays following the protocol outlined by Ball et al. (2007). A volume of 5 µL sample and standard DNA was added to a 96-well

PicoGreen™ microplate. A volume of 95µL of 1X TE lysis buffer solution and 100µL 200X PicoGreen™ working solution (Molecular Probes) were aliquotted to each well to make a final volume of 200µL per well. The samples were run on the automated robot FluoStar Galaxy unit using the BMG FluStar program. The plate reader delivered an excitation wavelength of 485 nm and measured the emission wavelength at 538 nm. The Relative Fluorescence Units (RFUs) were used to calculate the total DNA concentration and yield obtained from each extraction. As pointed out, PicoGreen™ estimates do not represent swift fox total DNA but rather a measure of all genomic DNA in the sample which could be from mammalian prey items or bacteria and/or parasites. Thus, further quantification of nuclear DNA was required to determine if the PicoGreen™ estimates represented actual swift fox DNA.

### **3.2.6 Target DNA Quantification**

Based on PicoGreen™ total DNA estimates, serial dilutions were prepared for all extracts using TE<sub>0.1</sub> to make the following concentrations: 1.25 ng/µL (5.0 ng), 0.25 ng/µL (1.0 ng), and 0.125 ng/µL (0.5 ng). Positive PCR control samples containing known quantities of swift fox DNA from swift fox tissue were diluted to 5ng, 1ng, 0.5ng, 0.2ng and 0.1ng per reaction. Negative PCR control samples were prepared while bovine serum albumen (BSA) was added to each reaction to sequester inhibitors and enhance amplification (Kreader 1996). Two polymorphic microsatellite loci of high molecular weight (HMW) of 300- base pairs (bp) and low molecular weight (LMW) of 200-bp were amplified using

VVE-M17 and VVE-M56 primers (Cullingham et al. 2007) PCRs were performed using 1X PCR buffer, 1.25 mM MgCl<sub>2</sub>, 0.1 mM dNTPs, 0.2 mg/mL BSA, 1U Taq DNA polymerase (Invitrogen), 0.3 µM of forward and reverse primers and 4µL template DNA in a final volume of 10 µL. The PCR cycling conditions included an initial denaturing step of 5 min at 94°C, 30 s at 55°C and 30 s at 72°C followed by 30 PCR cycles consisting of 30 s at 94°C of denaturing, 30 s at 55°C of annealing and 30 s at 72°C of extension, followed by one final cycle of 30 s at 94°C, 30 s at 55°C, 2 min at 72°C and maintained at 4°C until removal. Ten µL of each PCR product was electrophoresed on a 1.5 % agarose gel stained with ethidium bromide. The gel was run for approximately two hours at 100 volts, visualized under short-wavelength ultraviolet UV light and photographed using digital camera (Kodak 1D DC 290). Quantification of swift fox target DNA was based upon the positive control plateau at the established concentrations. That is, samples were scored based upon relative amplification band strength in comparison to the control sample. DNA quality, or level of DNA degradation, was estimated by comparing successful amplification of the smaller 200-bp fragment over the larger 300-bp microsatellite region (Ball et al. 2007). Presence of inhibitors was determined from the plateau test by observing band pattern at different concentrations. Quality of samples was also characterized by examining the relationship between PicoGreen<sup>TM</sup> estimates and the amplification success of the LMW and HMW target DNA.

### **3.2.7 Data analysis**

Amplification success rate of mtDNA and target DNA of summer vs. winter-collected samples were compared using Pearson's Chi-square test ( $\alpha < 0.05$ ) with Yates' continuity correction carried out for mtDNA amplification due to the low number of samples ( $< 5$ ) that did not amplify. The effect of mean daily temperature and maximum exposure on amplification rate was evaluated using logistic regression and the best model was selected using Akaike's information criterion (AIC). Amplification success of target DNA was measured as the amplification of one or two loci. Maximum exposure was estimated as the time that has elapsed from the time of defecation to the time of collection assuming that the animal had visited the bait station after 4 pm that evening (personal observation that animals were near den sites and on roads after 4 pm). Statistical analyses were carried out using S-PLUS version 6.2 (MathSoft, Inc. Cambridge, Massachusetts, USA) and SAS version 9.2 (SAS Institute, Inc., Cary, North Carolina, USA) for Microsoft Windows XP version 5.1.

## **3.3 RESULTS**

### **3.3.1 Species Verification**

Successful amplification and sequencing of mtDNA control region was achieved from 92% of selected samples (90% summer and 94% winter). Haplotype identification following direct DNA sequencing indicated that out of the samples that successfully amplified two were coyotes, one was red fox and the remaining 96% were swift fox. No significant difference was observed for mtDNA

amplification success among the two seasons ( $X^2 = 0.02$ ,  $P = 0.8875$ ,  $DF = 1$ ). Since the probability of a faecal sample originating from another canid was small (4.0%), in succeeding analyses the samples not tested for species identification were treated as originating from swift fox.

### 3.3.2 Quantification & Nuclear Amplification

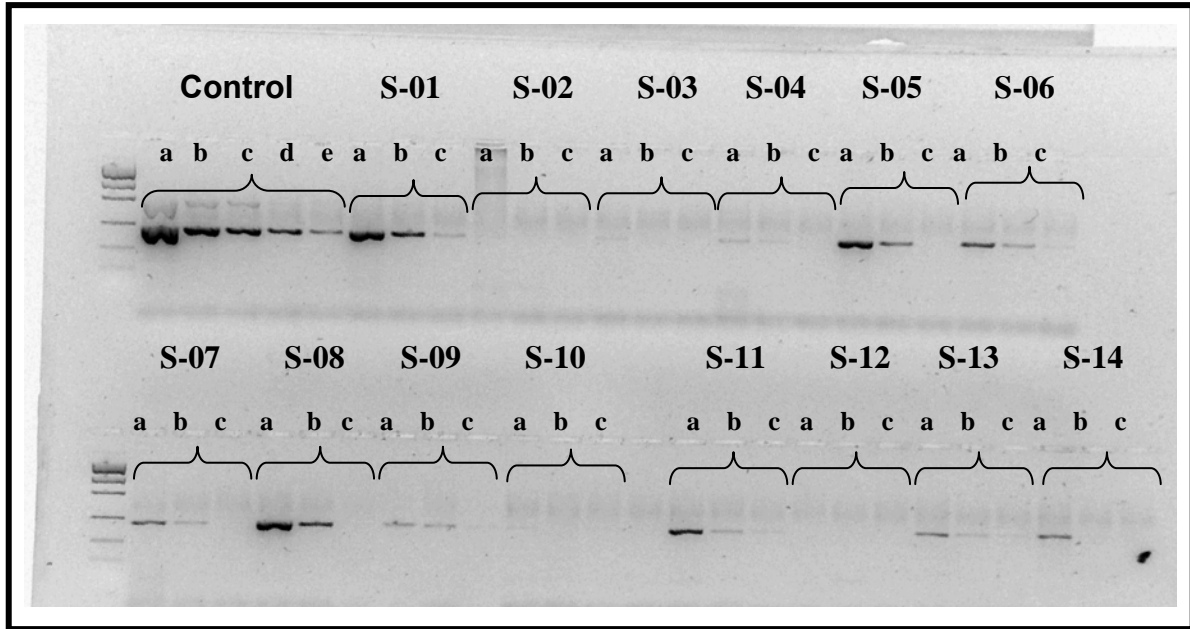
**Quantity:** Based on PicoGreen<sup>TM</sup> assays, total nuclear DNA concentrations for summer extracts was estimated to range from 0.74 - 33.85 ng/ $\mu$ L ( $X = 7.15$  ng/ $\mu$ L,  $SD = 8.30$ ) and 0.20 - 39.45 ng/ $\mu$ L ( $X = 12.40$  ng/ $\mu$ L,  $SD = 10.99$ ) for winter extracts. Mean total DNA yield per sample was 464.91 ng for summer-collected samples and 805.7 ng for winter-collected samples. Again, it is important to note that these estimates do not represent actual swift fox target DNA but rather total dsDNA present in the sample. The quantity of swift fox target DNA in each extract was estimated by comparing sample amplification band strength at three concentrations to the positive swift fox DNA control plateau at five concentrations (Figure 2). Swift fox target DNA in the range of 0.5 to 5.0 ng per reaction or greater, which is the range of template that minimizes allelic drop-out (Ball et al. 2007) and would be selected for simple genotyping protocol, was obtained from 25.3% and 47.3% of summer and winter extracts, respectively (Table 1).

**Quality:** A lower level of degradation was observed in the winter-collected samples since a greater number of samples successfully amplified the HMW



target DNA (68%) than the LMW marker (60.5%)(Table 2). Quality of samples was also characterized by examining the relationship between PicoGreen™ estimates and the amplification success of one microsatellite loci. Only 6.3% summer and 18.4% winter extracts showed the comparative amplification of  $\geq 5$ ng of target DNA indicative of a 1:1 total to target DNA ratio. For these samples, total nuclear DNA concentrations ranged from 3.85 to 33.85 ng/ $\mu$ L ( $X = 21.05$  ng/ $\mu$ L,  $n=4$ ) for summer samples and 1.32 to 34.5 ng/ $\mu$ L ( $X = 18.66$  ng/ $\mu$ L,  $n=14$ ) for winter samples. This comparison illustrates that a high proportion of both summer and winter-collected samples (93.7% and 81.6% respectively) did not correspond with PicoGreen™ estimates to produce a 1:1 ratio. Thus, it can be concluded that these samples contained some level of foreign DNA possibly from bacteria or consumed mammal species.

**Nuclear Amplification:** Based on a single locus amplification, winter-collected samples had a significantly higher nuclear amplification success (78.9%,  $n = 76$ ) than summer-collected samples (52.3%,  $n = 63$ )(  $X^2 = 10.98$ ,  $DF=1$ ,  $P < 0.001$ )(Table 2).



**Figure 2** - Amplification of HMW (300-bp) microsatellite loci from fourteen faecal extracts (S-01 to S-14) at three concentrations in comparison to swift fox DNA positive control at five known concentrations. Dark bands represent successful amplification of DNA seen in decreasing concentration. Lane a = 5.0 ng (1.25 ng/ $\mu$ L), b = 1.0 ng (0.25 ng/ $\mu$ L), c = 0.5 ng (0.125 ng/ $\mu$ L), d = 0.2 ng (0.05 ng/ $\mu$ L) and e = 0.1 ng (0.025 ng/ $\mu$ L).

**Table 1** - Quantification of swift fox target DNA, estimated from the amplification of 5.0 ng of total sample DNA and compared to control swift fox DNA at known concentrations. Sample size indicated in parentheses.

Sample Type	X ≥ 5.0 ng	5.0 ng > X ≥ 0.5 ng	X ≤ 0.2 ng	Total
Summer	6.3% (4)	19.0 % (12)	74.6 % (47)	100% (63)
Winter	18.4% (14)	28.9 % (22)	52.6 % (29)	100% (76)

**Table 2** – Quantification of quality of swift fox target DNA, estimated from the percent amplification of 2 loci of HMW and LMW.

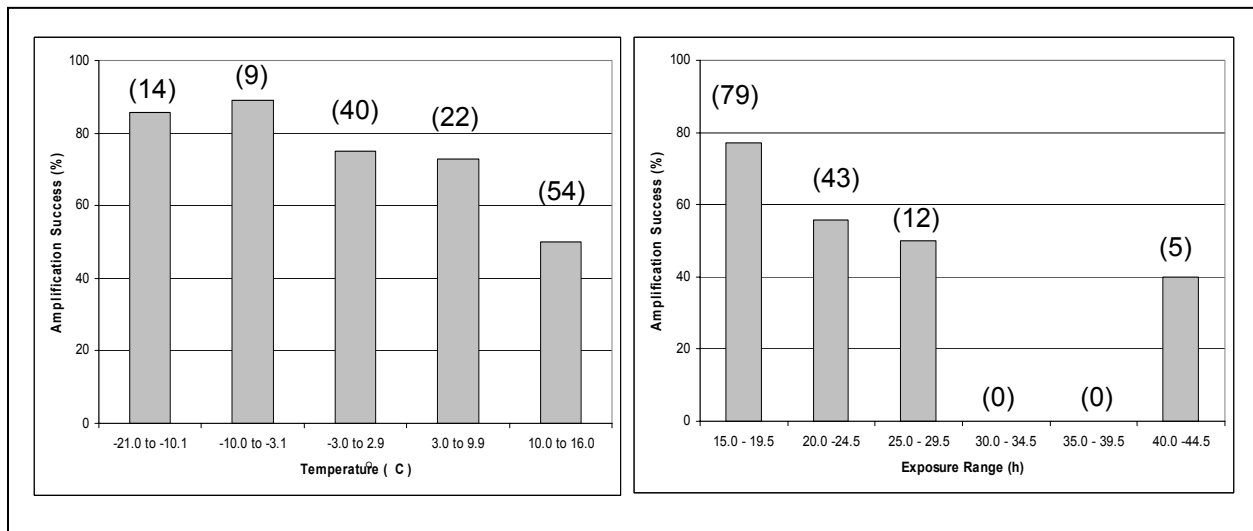
Sample Type	HMW (300 bp)	LMW (200 bp)	1 Loci HMW or LMW	2 Loci HMW & LMW
Summer (n=63)	42.9 % (27)	44.4 % (28)	52.3 % (33)	39.7% (25)
Winter (n=76)	68.4 % (52)	60.5 % (46)	78.9 % (76)	68.3% (43)

### 3.3.3 Factors Affecting DNA Amplification

During the sampling periods, mean summer ambient temperature was 11°C (range: 4.0 °C to 16.0°C) while winter mean temperature was -4°C (range: 0.0 °C to -19.0°C). The average time samples were exposed to environmental conditions during winter and summer was 19.9h (range: 15.5h to 42.5h) and 22.5 h (range: 17.5 to 27.5) respectively. Based on one locus amplification, nuclear amplification success decreased with increases in ambient temperature and exposure time (Figure 3). Because season and temperature were found to be highly correlated ( $r^2 = 0.82$ ), temperature was selected for regression analysis, as

this was a continuous variable and was considered more informative. A small correlation ( $r^2 = 0.36$ ) was also found between temperature and exposure; however, due to missing data for the exposure parameter (see Figure 3), the analysis was carried out. Logistic regression analysis revealed that both temperature and exposure models produce similar results. An Odds ratio of 0.90 was obtained for exposure under the exposure model and an Odds ratio of 0.93 for temperature under the temperature model (Table 3). The third model with exposure, temperature and an interaction term between exposure and temperature was not a better model with an AIC difference less than 4.

**Figure 3** - Percent of samples that amplified target DNA at one locus according to mean ambient temperature and maximum exposure on day of collection. Numbers in parentheses represent sample size.



**Table 3** - Results of the logistic regression models of one locus amplification success with ambient temperature and exposure as predictor variables (n=139).

Model	AIC	-2 LL	Estimate	Odds Ratio	SE	Wald 95% CI
Null	178.485	176.485				
Exposure	171.786	176.485	-0.107	0.898	0.040	0.830 – 0.972
Temperature	168.413	164.413	-0.074	0.929	0.023	0.888 – 0.972
Temperature & Exposure	166.741	160.741				

## 2.4 DISCUSSION

In this study, successful amplification and sequencing of mtDNA control region was achieved from 92% of selected samples which is much higher than those reported for other canids including coyotes (79% Kohn et al.1999, 84% Prugh et al. 2005) and kit foxes (*Vulpes macrotis mutica*)(76%, Smith et al. 2005). Of interest is that mtDNA amplification was not affected by seasonality since both summer and winter-collected samples had similar success rates. This finding suggests that for species identification sample collection can be carried out in either summer or winter conditions.

Based on the amplification of one locus, successful amplification was achieved from 52% summer and 79% winter-collected samples (Table 2). The significantly higher amplification rate observed in the winter-collected samples illustrates that seasonality does play an important role in nuclear amplification. Improved success in nuclear amplification rates for winter-collected over summer

or spring-collected samples have also been reported for wolf and ungulate species (Lucchini et al. 2002; Maudet et al. 2004), although Maudet et al. (2004) attributed this seasonal difference to “variations in vegetation and diet quality”. While swift fox seasonal diet quality was not evaluated in this study, Broquet et al. (2007) did assess several faeces-based studies and concluded that the amplification rate was not influenced by the diet of the target species.

In this study, higher amplification success was observed to be influenced by temperature. That is, winter-collected samples had a higher amplification success, with lowest amplification rates observed for samples collected above 10°C. Based on AIC model results, temperature was a significant parameter in explaining DNA amplification success. The higher amplification rates recorded in winter-collected samples are believed to be influenced by the low temperatures which reduce the DNA degradation rate by digestive enzymes (Nsubuga et al. 2004; Prugh et al. 2005; Hajkova et al. 2006). Consistent with these results, Hajkova et al. (2006) and Nsubuga et al. (2004) reported that temperature at the time of faecal collection negatively affect DNA quantity of faecal samples collected from wild otters and apes.

Furthermore, in this study, winter-collected samples produced higher quantity of swift fox target DNA than summer samples. That is, almost half of the winter –collected samples (~47%) produced yields of target DNA in the range of 0.5 to 5.0 ng or greater suggesting that these samples had higher quantities of amplifiable nuclear DNA available for simple genotyping protocols. Although the remainder of samples did not pass the 0.5 ng threshold, further amplifications

could be carried out to obtain target DNA or the samples can simply be removed from data set thus avoiding genotyping errors (Ball et al. 2006; Scandura et al. 2006). In addition, the quality of swift fox target DNA extracted from winter-collected faecal samples was better than the summer samples. By comparing total DNA estimated by PigoGreen assays to amplifiable target DNA, a higher proportion of winter-collected samples having a 1:1 ratio was found, thus indicating a lower level of degradation or foreign DNA in winter-collected faecal samples.

Microsatellite amplification results reported in this study are difficult to compare with those from other canid studies since only two loci were amplified and only one PCR reaction was carried out per locus. That is, most faecal-based studies use several microsatellite loci and employ the multitubes approach where several PCRs (minimum 8 and up to 20 reactions) are carried out per locus (Taberlet et al. 1996) in order to achieve successful amplification and reliable genotypes. As such, canid amplification success rates vary significantly across studies. For example, amplification success was reported to range from 53% (6 loci)(Lucchini et al. 2002) to 79% (13 loci) (Creel et al. 2003) for gray wolves (*Canis lupus*), 50% (6 loci) for red wolves (*Canis rufus*) (Adams & Waits 2007), 79% (6 loci) for kit foxes (Smith et al. 2005) and as much as 100% (6 loci) for captive-raised red foxes (Piggott & Taylor 2003).

Although nuclear amplification was carried out on only two loci, the higher amplification success and quantity of target DNA obtained in this study is attributed to several factors. First, only the outer mucous layer of the faecal

sample was utilized for DNA extraction (Ball et al. 2007). This technique contrasts with traditional extraction methods where a small amount (eg. 180-220 mg) of whole faecal material is homogenized in buffer solution prior to DNA extraction (Paxinos et al. 1997; Harrison et al. 2002; Creel et al. 2003; Prugh et al. 2005; Smith et al. 2005). This protocol assumes that target DNA is found throughout the faecal matter in the form of shed epithelial cells. Wehausen et al. (2004) reported higher amplification rates for extracts where scrapings from the outer layer of sheep faecal pellets were used rather than for extracts where the inner material was included. This protocol assumes that the outer layer of the faecal sample contains a significant amount of high quality DNA originating from the intestinal tract of the target species and contamination from PCR inhibitors and foreign DNA found throughout the faecal sample are minimized (Flagstad et al. 1999; Fernando et al. 2003; Maudet et al. 2004; Ball et al. 2007). The presence of PCR inhibitors was also minimized by using a silica-based commercially purification kit, in combination with an ethanol wash, which have been found to be very effective in reducing inhibitors (Wasser et al. 1997; Ye et al. 2004; Ball et al. 2007). Lastly, serial dilutions, or plateau tests, were carried out for each extract and viewed on agarose gel to assess whether PCR inhibitors are posing a problem.

By using fresh faecal samples, verifying the species through the amplification of mtDNA control region followed by microsatellite amplification and target DNA quantification, this study verified that winter-collected samples have a higher amplification success and contain better quality and quantity swift fox



target DNA than summer-collected samples. Furthermore, temperature was found to be a significant factor in microsatellite amplification success thus it is recommended that swift fox faecal sample collection be conducted in winter months when temperatures fall below freezing and DNA degradation is minimized. Although further analysis is required in establishing genotypes and quantifying genotyping error rates for each season, this study illustrates that fresh winter-collected swift fox faecal samples can produce adequate target DNA in the range that minimizes genotyping error and be used for simple genotyping protocols.

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## **CHAPTER 4**

### **Estimating swift fox abundance using noninvasive faecal DNA sampling: Comparison of two field survey methods**

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#### **4.1 INTRODUCTION**

The swift fox is Canada's smallest canid species which inhabits the native shortgrass prairies of Saskatchewan and Alberta and is currently listed by the Committee On the Status of Endangered Wildlife In Canada (COSEWIC) as endangered (COSEWIC 2000). Historically, the swift fox ranged throughout the shortgrass and mixed-grass prairies of North America but intense trapping, predator and rodent eradication poisoning programs, in combination with habitat loss due to conversion of native grassland to farmland, significantly reduced this species distribution such that only small isolated populations remained (Banfield 1974; Egoscue 1979; Carbyn 1998; Sovada et al. 2001). By the late 1920s, the swift fox had entirely disappeared from Canada and in 1978 COSEWIC officially designated the swift fox as extirpated (Carbyn 1998). Intensive captive breeding, translocation, and reintroduction programs between Canada and the United States involving federal and provincial governments, universities, non-government organizations and landowners, resulted in the successful establishment of a small swift fox population on the Canadian prairies (Carbyn 1998; Moehrensclager & Moehrensclager 2001) and in 1998 the species was

down listed to the endangered status (COSEWIC 2000). While the Canadian population has been recently estimated to be around 647 individuals and stable (Moehrenschlager & Moehrenschlager 2006), conservation and monitoring efforts are still needed given that population threats such as habitat loss and degradation, high mortality rates due to predation and car collisions, and possible disease outbreaks, continue to negatively affect the status of this species (Moehrenschlager & Sovada 2004; Pruss et al. in review).

Population size and geographic distribution are important population parameters central for effective management and recovery plans of an endangered species however, such data are difficult to obtain, particularly for species like the swift fox which are nocturnal, elusive, rare and exist at low density (Ernest et al. 2000; Creel et al. 2003; Smith et al. 2005). The current method of obtaining population data is by the traditional live-trapping which is expensive, time consuming, and most of all poses risk of injury and distress to the animals (Kohn et al. 1999; Harrison et al. 2002; Smith et al. 2005). Minor injuries in the form of scrapes, chipped teeth, broken mandible or claw sheaths, and paw laceration have been encountered in live trapping surveys (Schauster et al. 2002; Moehrenschlager et al. 2003). Noninvasive methods such as scent-stations, scat counts, track counts and spot-lighting have been used to measure relative abundance and/or occupancy range (Harrison et al. 2002; Finley et al. 2005; Sargent et al. 2005; Smith et al. 2005), but no single technique has yet been established to estimate absolute abundance in Canada or the United States for the swift fox. The development of alternative noninvasive survey

methods has been identified by the World Conservation Union (IUCN) Canid Conservation Group (Sillera-Zubiri et al. 2004) and the Swift Fox Recovery Team as a priority for the recovery and conservation of the swift fox on the Canadian prairies. Clearly, a standardized rigorous noninvasive technique to accurately assess population abundance and to phase out the live-trapping method is highly desirable.

Recent population studies have demonstrated that faecal material is a reliable source of DNA for individual identification and population estimation (Kohn et al. 1999; Eggert et al. 2003; Wasser et al. 2004; Bellemain et al. 2005; Ball et al. 2007) and as such can be applied to monitor populations over time (Prugh et al. 2005). Faecal material can be easily obtained from wild individuals especially from canids which tend to defecate along roads and trails as well as on conspicuous objects to mark territories and communicate with each other (Sharp et al. 2001; Barja et al. 2004; Smith et al. 2005). Swift foxes in particular are known to be curious and to mark novel object such as sent-stations with scat or urine (Harrison et al. 2002; Shaughnessy 2003). The majority of canid studies utilizing faecal material for genetic analysis have used samples of unknown or several days of age, targeting a large sample size (Kohn et al. 1999; Harrison et al. 2002; Adams et al. 2003; Prugh et al. 2005; Smith et al. 2005). However, sample age has been found to significantly affect nuclear amplification with freshly collected samples showing higher amplification success and lower genotyping error rates than older samples (Lucchini et al. 2002; Piggott 2004).



Collecting fresh (<1day) faecal samples from wild swift foxes for the purpose of DNA sampling and individual identification has not previously been reported.

Many researchers have cautiously pointed out that incorrect identification of individuals, or genotyping error, routinely occurs when working with non-invasive samples where template DNA is limited in quantity and quality (Gerloff et al. 1995; Taberlet et al. 1996; Pompanon et al. 2005). To obtain reliable genotypes, the multiple-tubes approach developed by Taberlet et al. (1996) has been adopted in noninvasive studies as the standardized method to reduce genotyping errors. However, this method can be expensive, time consuming, and unrealistic for large scale studies (Flagstad et al. 1999; Fernando et al. 2003). Screening of samples, by rejecting poor-quality samples from genotyping, has been shown to decrease genotyping error rates and reduce laboratory costs and effort (Morin et al. 2001; Ball et al. 2007).

Faecal DNA genotyping has the potential to become a practical and effective population survey technique for the endangered Canadian swift fox if fresh faecal samples from a high proportion of the population can be effectively collected in the field and genetically analyzed. Thus, the goal of this study was to examine the feasibility of faecal genotyping as a noninvasive population survey technique for the Canadian swift fox by testing two field methods of collecting faecal samples in both high and low density areas in three different seasons and genotyping samples to the individual level to determine the number of individuals that can be captured in the study area. To reduce genotyping error rates, the quantification method developed by Ball et al. (2007) was adopted where

samples were screened and only those with high quantity and quality swift fox target DNA were genotyped. Overall, this study can determine if faecal DNA genotyping has the potential of becoming an effective sampling method for determining swift fox population size in Canada.

## **4.2 METHODS**

### **4.2.1 Study Area**

The study was conducted in the native grasslands of southwestern Saskatchewan, north of the Montana border and east of the Alberta border, on federal land managed by the Prairie Farm Rehabilitation Administration (PFRA) (Figure 1). Captive-raised and translocated swift foxes have been re-introduced in this area from 1983 to 1997 and population monitoring has been carried out in the area since the last reintroduction (Cotterill 1997; Moehrensclager & Moehrensclager 1999; Moehrensclager & Moehrensclager 2001, 2006). The study site, spanning an area of 565 km<sup>2</sup>, consisted of six townships which are exclusively utilized for cattle grazing; however, gas exploration activities, such as drilling, storage, transportation, and road building, as well as recreational hunting and scientific research took place in the area. Agricultural activities occurred on the north and west periphery of the study area while cattle grazing pastures were present to the east and south of the study site. The site has been designated as a nationally Important Bird Area (IBA) supporting a rich diversity of endangered and vulnerable bird species including the burrowing owl (*Athene cunicularia*),

greater sage grouse (*Centrocercus urophasianus*), ferruginous hawk (*Buteo regalis*), and sprague's pipit (*Anthus spragueii*) (Schmutz 2000). Selection of the study area was primarily based on accessibility and density of foxes. Based on the published 2000-2001 swift fox live-trapping population census data, swift fox density varied among the selected townships thus a classification method was designed for the purpose of this study. Govenlock, Nashlyn 1, and Nashlyn 2 were considered high density townships with equal or greater than 4 unique foxes captured during the trapping session ( $X = 6$  foxes/township), while Battle Creek 1, Battle Creek 2, and Battle Creek 3 were considered low density townships with equal or less than two foxes captured ( $X = 2$  foxes/township) (Moehrensclager & Moehrensclager 2001). The six townships were connected to reduce traveling time and were treated as unique sampling units.

Regional climate is representative of the continental prairies characterized by hot dry summers and cold, harsh winters. Maximum temperature can range from as high as 40°C to as low as - 43°C; annual precipitation levels are approximately 270 mm with summer season receiving about 45 mm per month (Environment Canada 2005). The topography varies from flat prairies to gentle rolling hills including coulees and creeks. The predominant ecosystem is the native shortgrass prairie which is composed of the *Stipa-Bouteloua-Agropyron* vegetation type characteristic of the region's shallow sandy loam soils (Moehrensclager 2000). The dominant grass species are needle and tread (*Stipa comata*), blue grama (*Boutela gracilis*), northern wheatgrass (*Agropyron dasystachyum*) and western wheat grass (*Agropyron smithii*) but forbs and shrub

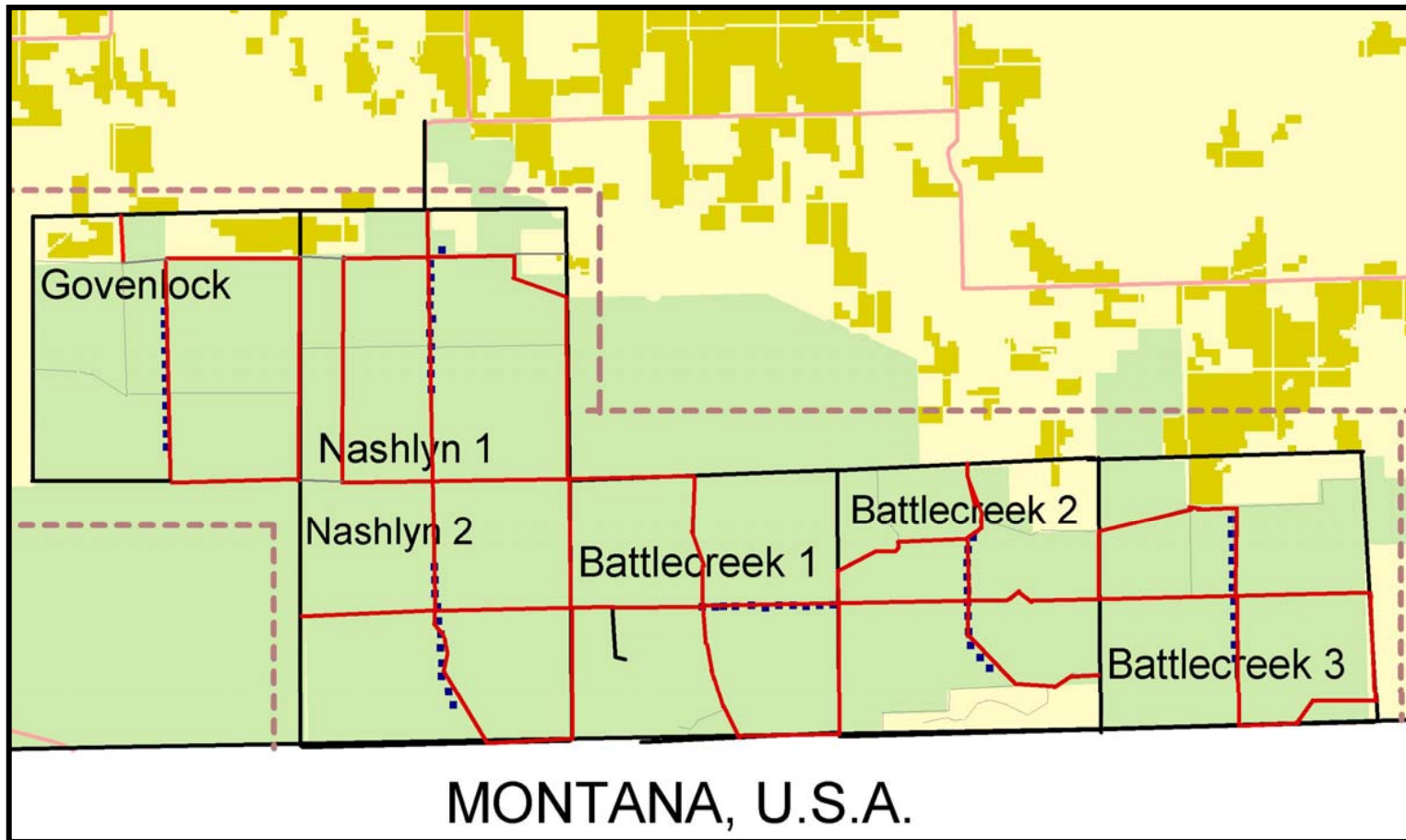
species such as moss phlox (*Phlox hoodii*), broomweed (*Gutierrezia sarothrae*), pasture sage (*Artemisia frigida*), winterfat (*Eurotia lanata*), salt sage (*Artiplex muttallii*) and sagebrush (*A. cana*) are also common (Moehrenschrager 2000). Elevation ranged from 850 m to 1050 m above sea level (Moehrenschrager 2000).

#### 4.2.2 Field surveys

Canid-like faecal samples were collected in late summer (August-September) and early winter (November-December) of 2005, and late fall (November) of 2006 to determine the optimum season to conduct the surveys. Two survey methods were tested in both the high and low population density areas to establish an effective method of recovering fresh swift fox faecal material from a high proportion of the population for the purpose of extracting DNA and individual identification.

**Method 1: Roads/trails** - Dirt roads and grass trails were systematically searched for canid faecal material using all-terrain vehicles (ATVs) driven at a maximum speed of 10-km per hour (Adams et al. 2003) in both summer and winter of 2005 (Figure 4). The survey method was not carried out in the fall of 2006. Prior to the survey, the established route was searched and all scats were removed in order to ensure that subsequent faecal samples were fresh (< 1 day). The same route was searched for 4 consecutive days and all encountered canid-like faecal material was collected in sterile labeled whorl plastic bags and placed in a small cooler containing two frozen ice packs (Plate 1). To avoid cross-

sample contamination, a new stick was used to collect each sample, and scats located further than 5 cm apart were treated as separate samples. The location of each sample was geo-referenced with a hand-held GPS unit (Garmin 12 XL) and specific sample data such as date, time, location type, freshness, and species of origin were entered in a Personal Data Assistant (PDA) (Trimbel GeoXM or Pocket PC Recon) using the CyberTracker software (*for type of data collected see Appendix 1*). Samples with a diameter  $\geq 20$  mm were classified as coyotes (*Canis latrans*) (Harrison 2003) while those with a diameter  $< 20$  mm and possessing morphological characteristics of a small canid were categorized as fox (possibly swift or red fox, *Vulpes vulpes*). Scats that appeared to be canid-like but could not be accurately identified as either fox or coyote were also collected and classified as “unknowns”. At the end of the day, samples were transferred to a freezer exclusively used for the storage of canid faecal material collected during this survey and stored without preservatives at temperatures below 20°C (Creel et al. 2003). Due to the accumulation of deep snow, two low density townships (Nashlyn 2 and Battlecreek 3) were inaccessible during the winter period thus they were not surveyed at that time.



**Figure 4** – Map of survey routes in summer and winter of 2005 and fall 2006. Roads/trails method survey route is represented in red continuous lines. Bait –stations transects are represented in blue squares.



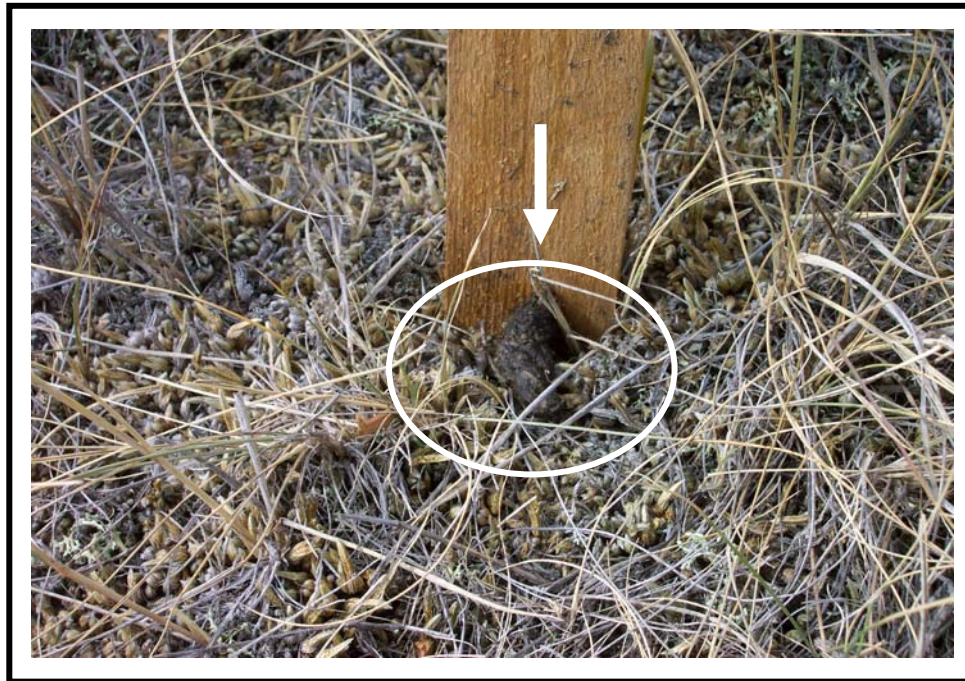
**Plate 1.** A) Surveyor using the roads/trails method; B) Swift fox scats found on the road; C) Surveyor collecting swift fox faecal samples.

**Method 2: Bait-station.** To determine if scat recovery rate can be increased, surveys using bait-stations were conducted in summer and winter of 2005 and fall of 2006 replicating carnivore scent-station techniques as outlined by Sargeant et al. (2003) and Uresk et al. (2003). It is believed that baiting an area will not affect swift fox predation risks (Moehrenschrager personal communication); thus the technique is believed to be safe for swift foxes. Paralleling the live-trapping population census protocol, bait-stations were established at 0.5-km intervals along a 5-km transect for a total of 11 bait-stations per township which were placed within the center of each township to capture as many individuals as possible within the township (Figure 4). Although evening traffic is minimal in the study area, bait-stations were placed a minimum of 10.0 m away from the road to avoid animal vehicle collisions. Each station consisted of a wooden stake wedged in the ground and baited with approximately 20 g canned sardines (Uresk et al. 2003) which were placed on top of the stake to increase scent dispersal and to keep bait from beetles. The stakes were also saturated with the sardine–oil mixture to attract individuals to the bait-station in the event that the bait was consumed by other animals. Bait stations were set up on the first day and revisited for 4 consecutive days. Each day, the stations were re-baited with fresh sardines and oil and a radius of 1-m was searched for possible scats (Sharp et al. 2001). All encountered canid scats were collected and stored as previously described (Plate 2).

Winter and fall surveys were carried out in similar a manner as the summer survey with a few exceptions. During the summer, the 5-km road



paralleling the bait-station transect was also surveyed with the ATV with the goal of increasing sample recovery rate. However, only 13% of total samples were collected using this method; therefore, to simplify the survey, this method was not repeated during the winter or fall. In addition, in both the winter and fall surveys, the ATVs were replaced with a truck which was found better suited for the low temperatures and deep snow. During the winter survey, 3 transects had to be re-located within the same township since they became inaccessible as a result of accumulation of deep snow on the roads while during the fall survey 4 transects were not checked on the last survey day due to a severe blizzard.



**Plate 2.** Swift fox scat deposited at bait-station during: A) summer and B) winter survey period of 2005 in southwestern Saskatchewan.

### 4.2.3 Genetic Analysis

Upon completion of all surveys, all samples were transported to Winnipeg, Manitoba, in coolers packed with ice packs and stored in a freezer at  $-20^{\circ}\text{C}$ . With the exception of the scats classified as coyote, all samples were sent to the Natural Resources DNA Forensics and Profiling Centre (NRDFPC) lab at Trent University, Ontario, for DNA extraction and processing. Following the completion of the field surveys, the effectiveness of the two sampling methods was quickly apparent. Therefore, genetic analysis efforts were primarily placed on samples collected using the bait-station method. Samples collected during fall of 2006 have not yet been processed. DNA was extracted from 63 summer and 76 winter-collected faecal samples using the QIAam DNA Tissue Kit using spin columns following manufacturer's protocol (*see Chapter 3*).

**Sample Screening:** To avoid the need of carrying out multiple locus amplifications (ie. multi-tubes approach) and to reduce genotyping errors which are associated with DNA extracted from faecal samples (Taberlet et al. 1996), target DNA was quantified prior to species identification and genotyping to determine which extracts successfully amplified swift fox microsatellite DNA, which samples produced high quality target DNA equal or greater than 0.5 ng, and whether PCR inhibitors were a problem. Two canid tetranucleotide microsatellite markers (VVE2-64 and VVE2-110), one low molecular weight (LMW) of approximately 200 base pairs (bp), and one high molecular weight (HMW) of approximately 300-bp (Cullingham et al. 2007), were amplified following the protocol described by Ball et al. (2007) and as outlined in Chapter 3.

**Species Verification:** To determine if samples collected at the bait station did not originate from other sympatric species such as red foxes, coyotes, or badgers (*Taxidea taxus*), species verification was carried out on a subset of 77 (~ 50%)(30 summer and 47 winter). Out of these, 26 samples represented extracts which did not amplify target DNA at either of the two LMW and HMW loci during the screening process. These 26 samples were considered problematic and during the screening process they would have been rejected due to high risk of genotyping errors. Species identification and haplotype were achieved by amplifying a portion of the mtDNA control region using the forward primer – L15997 and reverse primer – H16498 (Kocher et al. 1989)(see Chapter 3).

**Genotyping:** Based on the screening process, samples that produced high quantity target DNA (> 0.5 ng) and were confirmed to originate from swift fox were selected for genetic profiling. Genotyping was carried out on 46 samples (16 summer and 36 winter) using nine polymorphic tetranucleotide microsatellite markers (VVE3-131, VVE5-45, VVE2-64, VVE-M19, VVE-M17, VVE-M25, VVE2-111, VVE2-110) with an estimated mean heterozygosity of 0.74 (range: 0.417 to 1.00) (Cullingham et al. 2007). To reduce cost and effort, the 26 problematic samples were only analyzed at five loci (VVE2-64, VVE5-33, VVE-M19, VVE-M25, VVE2-110) as an initial screening. For all samples, each loci was amplified independently using the protocol described by Cullingham et al. (2007) with the exception of primer conditions which were increased to 0.35 $\mu$ M and the number of cycles adjusted from 30 to 35. All samples were profiled on the MegaBASE1000 system (GE Healthcare) and raw data processed in Genetic

Profiler (GE Healthcare). To determine if each sample represented a unique individual, probability of identity (PI), which is defined as the probability of two individuals in a population carrying the same genotype by chance, (Mills et al. 2000), was calculated for each locus using GenAlEx (Peakall & Smouse 2006).

#### **4.2.4 Statistical Analysis**

Scat recovery per unit effort was calculated as the total number of faecal samples collected divided by the number of days that the survey took per work unit which consisted of one vehicle (ie. an ATV or a truck) and at least one surveyor. For the bait-station method, scat deposition rate was calculated as the percent of total number of samples collected divided by the number of operational scent-station nights (Reed 2000). The effectiveness of both methods in locating faecal samples in both high and low density townships was also compared. As an index of accuracy, a paired t-test (S-PLUS version 6.2, MathSoft, Inc. Cambridge, Massachusetts, USA)( $\alpha < 0.05$ ) was used to test for significant difference between the number of individuals captured in a given township using the noninvasive bait-station method and the number of live individuals trapped in the same study area during the winter of 2005-2006 Swift Fox Population Census. Since the live trapping population census took place during the winter period, only the genotypes generated from the winter-collected samples were compared.

## **4.3 RESULTS**

### **4.3.1 Sample Collection**

A total of 559 canid-like faecal samples were collected during the three survey periods from both high and low density townships (Table 4). Based on morphological characteristics 14% (81/573) were classified in the field as originating from coyote therefore these samples were excluded from the data set. Out of the total 478 fox-like samples collected, 57% and 32% of the samples were collected during the summer and winter, respectively. Since the fall survey period consisted of the bait-station method, only 11% of the samples were collected during this season. Across all seasons, an average of 24.6 and 11.7 faecal samples were collected per township using the roads/trails and bait-station methods respectively. However, since almost all six townships were surveyed in one day using the bait-station method, across all seasons sample recovery rate was much higher for the bait-station method ( $X=9.8$  samples/day) than the roads/trails method ( $X=5.1$  samples/day) (Table 5).

**Table 4** – Summary of total and mean number of fox-like faecal samples collected per township by each method during summer and winter of 2005 and fall of 2006.

Township	Roads/Trails		Bait-station		
	Summer	Winter	Summer	Winter	Fall
1. Govenlock	42	18	22	9	1
2. Nashlyn 1	66	31	21	34	19
3. Nashlyn 2	17	2	8	10	15
4. Battlecreek 1	36	6	9	5	16
5. Battlecreek 2	25	N/A	4	11	3
6. Battlecreek 3	24	N/A	2	10	1
Total	210	57	66	90	55
Mean	35.0	14.3	11.0	15.0	9.2

**Table 5** – Sample recovery rate of fox-like faecal samples per unit effort (sample/day) by each method during summer and winter of 2005 and fall of 2006 (N=6 townships).

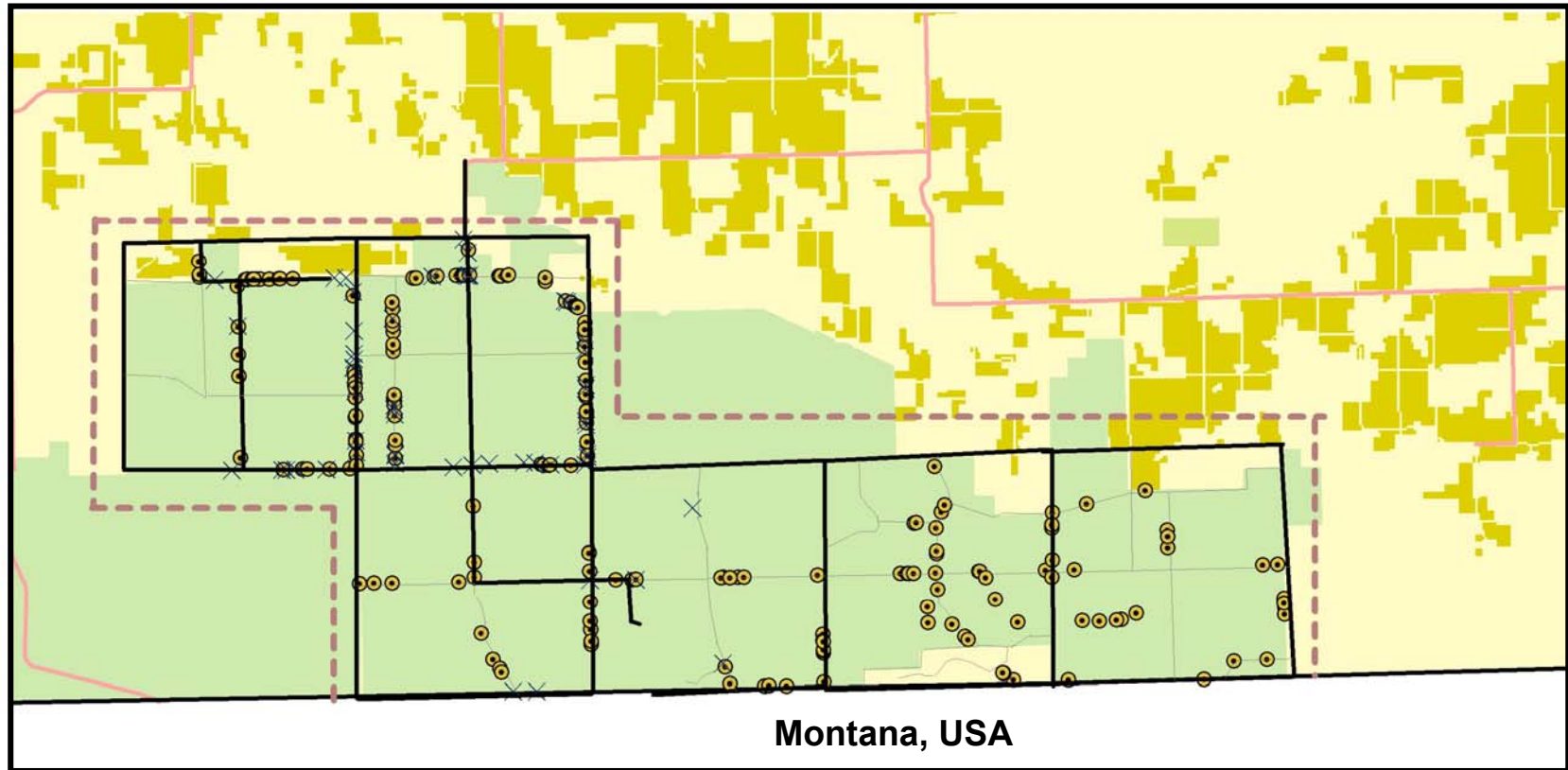
Season	Roads/Trails	Bait-station
Summer	7.0	9.4
Winter	3.2*	9.0
Fall	N/A	11.0
Mean	5.1	9.8

\* N=4

**Roads/Trails Method:** Fresh fox-like faecal samples were collected daily during both summer and winter sampling periods (Figure 5). Two townships were not surveyed during the winter as a result of severe winter conditions and road access. Overall, a higher proportion of faecal samples were collected during the summer survey ( $X=35.0$  scats/township) than during the winter survey ( $X=14.3$  scats/township). An average of 8.8 samples (range: 3 to 23) were collected per township per day in summer, while in winter, an average of 4.8 samples (range: 1

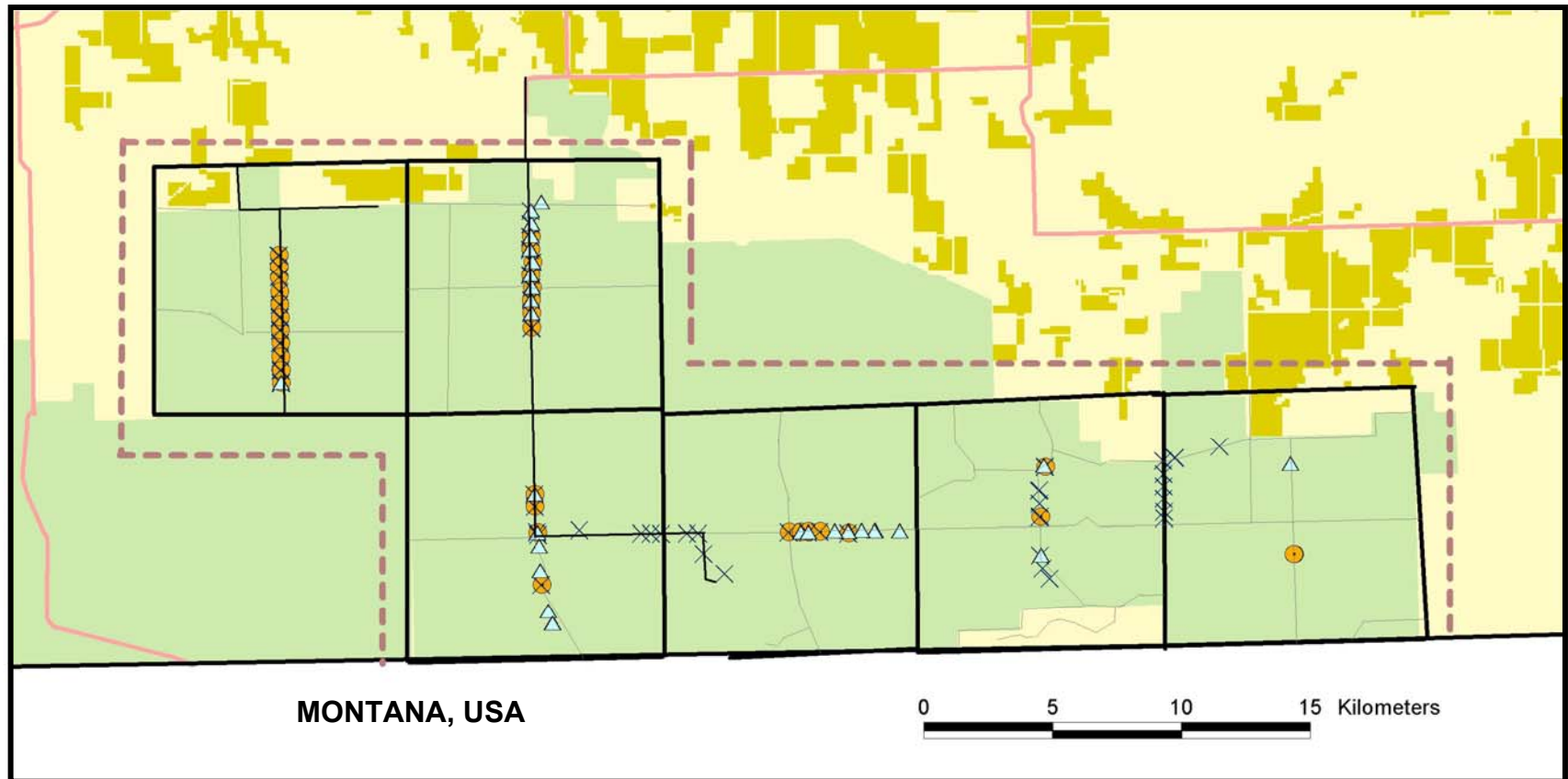
to 13) were collected per township (Table 6). Depending on the availability and accessibility of roads and trails in each township, an average of 34.5 km (range 27 to 40 km/township) were surveyed daily per township for a total of 828.4 km for the summer survey and 525.6 km for the winter survey. Based on morphological characteristics, 90% and 79% of total faecal samples collected in summer and winter respectively were classified as originating from a fox. Daily surveys took an average of 6 hours (range: 4.9 to 8.3 hours), thus only one township could be surveyed per person per day. Summer and winter surveys took 15 days to complete.





**Figure 5** – Fox-like samples collected using the roads/trails method during the summer (orange circles) and winter (blue exes) surveys in 2005.

**Baiting method:** Fresh fox-like faecal samples were collected from all of the townships in all three seasons (Figure 6). A higher proportion of faecal samples were collected during the winter survey ( $X = 15.0$  samples/township) than during the summer ( $X=11.0$  scats/township) or fall (9.2 scats/township) surveys (Table 1). Per day, an average of 2.8 faecal samples (range: 0 to 9) were collected per township in summer, while in winter 3.9 (range: 0 to 17) and in fall 2.9 samples (range: 0 to 10) were collected (Table 6). During the summer survey, faecal samples were successfully collected from 45% of the total bait stations established (6 transects x 4 sampling days), 71% during winter, and 41% during fall. For all seasons, an average of 0.5 scats were collected at each bait station (range: 0 to 4 scats/station). Based on morphological characteristics, only 3.0% of scats collected at the bait-station were classified as “unknown”. Scat deposition rate (total scats / scent-station night) was highest for the winter survey (32%) but similar for the fall and summer surveys (app. 25%). Setting up a transect (ie. 11 bait-stations) took between 1 to 1.5 hours depending on the distance of the points from the road, while checking a transect took an average of 1.4 hours depending on how many samples were encountered. The summer survey took 7 days to complete while the winter survey took 10 days due to inaccessible roads resulting in the relocation of 3 transects and starting the survey again. The fall survey took 5 days to complete.



**Figure 6** – Fox-like samples collected using the bait-station method during summer represented by orange circles, winter represented by blue exes, and fall represented by blue triangles.

**Table 6** - Mean number and  $\pm$  standard deviation of fox-like faecal samples collected per township on the first, second, third, and fourth day of survey during summer and winter 2005 and fall 2006 in southwestern Saskatchewan.

Day	METHOD						
	Roads/Trails			Bait-station			
	Summer	Winter	Mean/day	Summer	Winter	Fall	Mean/day
<b>1</b>	7.0 $\pm$ 5.5	3.7 $\pm$ 2.6	5.7 $\pm$ 4.7	2.2 $\pm$ 1.8	1.3 $\pm$ 0.5	1.8 $\pm$ 2.1	1.6 $\pm$ 1.7
<b>2</b>	11.8 $\pm$ 7.3	3.0 $\pm$ 3.6	8.3 $\pm$ 7.4	2.8 $\pm$ 3.8	4.2 $\pm$ 3.8	4.7 $\pm$ 4.8	3.5 $\pm$ 3.8
<b>3</b>	9.3 $\pm$ 3.6	5.5 $\pm$ 5.4	7.8 $\pm$ 4.6	2.5 $\pm$ 2.9	3.3 $\pm$ 2.5	2.7 $\pm$ 2.3	2.8 $\pm$ 2.4
<b>4</b>	6.8 $\pm$ 2.8	4.0 $\pm$ 0.0	6.1 $\pm$ 2.7	3.5 $\pm$ 2.1	7.4 $\pm$ 5.5	2.0 (NA)*	5.0 $\pm$ 4.2
<b>Mean scats/day</b>	<b>8.8 <math>\pm</math> 5.2</b>	<b>4.8 <math>\pm</math> 3.3</b>		<b>2.8 <math>\pm</math> 2.6</b>	<b>3.9 <math>\pm</math> 3.9</b>	<b>2.9 <math>\pm</math> 3.8</b>	

\*n=1.5 transects were surveyed.

**Density:** As expected, a higher proportion of faecal samples were collected in high density townships than in low density townships (table 7). During the summer survey, a greater number of samples were collected using the roads/trails method than the bait-station method, particularly from the low density townships; however more samples were collected during the winter survey using the bait-station method than using the roads/trails method. Since species verification was not carried out on any of the roads/trails samples, it is not known if all the samples collected using this method originated from swift foxes.

**Table 7** – Total number and  $\pm$  standard deviation of fox-like faecal samples collected per day in high and low density township in each season in Southwestern Saskatchewan (N=3 townships).

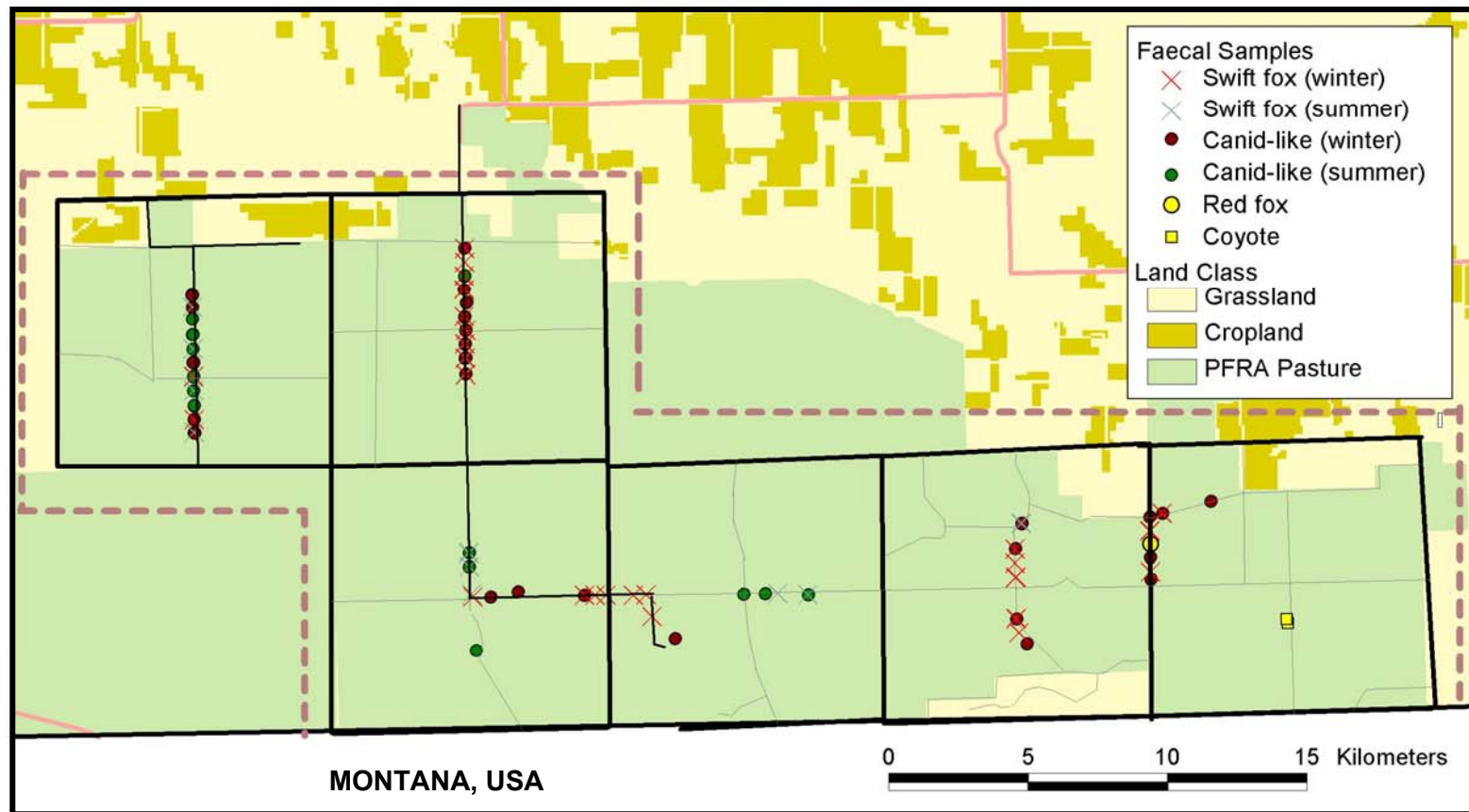
Season	Roads/Trails		Bait-station	
	High density (N=3)	Low density (N=3)	High density (N=3)	Low density (N=3)
Summer	10.4 $\pm$ 6.3	7.1 $\pm$ 3.3	4.3 $\pm$ 2.5	1.3 $\pm$ 1.8
Winter	4.6 $\pm$ 3.2	2.0 $\pm$ 2.0*	5.3 $\pm$ 4.9	2.5 $\pm$ 1.3
Fall	NA	NA	3.5 $\pm$ 3.1	2.2 $\pm$ 3.2
Overall mean	7.3 $\pm$ 5.6	6.1 $\pm$ 3.7	4.4 $\pm$ 3.7	1.9 $\pm$ 2.2

\* N=1 township.

#### 4.3.2 Sample Screening and Species Verification

High quality target DNA in the range of 0.5 to 5.0 ng or greater, which is required for simple genotyping protocols (Ball et al. 2007), was obtained from 26 (25.3%) and 36 (47.3%) summer and winter extracts, respectively (see *Chapter 3*). Successful amplification of mtDNA control region was achieved from 92% of

the selected samples. Haplotype identification indicated that two were coyotes, one was red fox, and the remaining (96%) samples originated from swift fox. Out of the 26 problematic samples, 81% successfully amplified mtDNA, one being a coyote sample. Two different haplotypes (WYO13 and SD1) were found in the sampled population (haplotype designations based on Maldonado et al. 1997). Based on this analysis, swift fox samples were recovered from all of the six surveyed townships while the three non-swift fox samples were all collected from Battlecreek 3, a low density township (Figure 7).



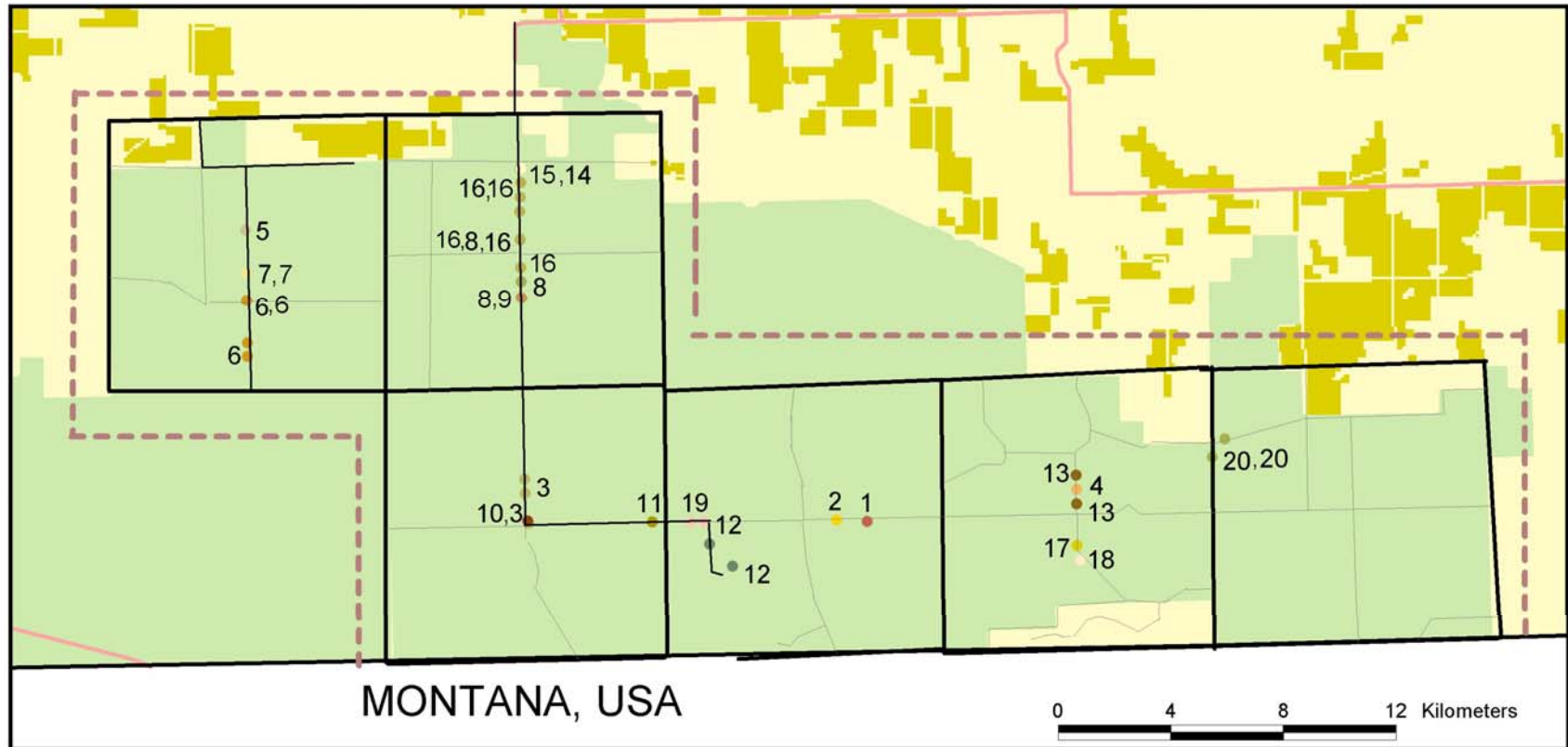
**Figure 7** – Distribution of swift-fox and canid-like faecal samples collected in summer and winter of 2005. Swift fox faecal samples were identified to species using mtDNA control region.

### 4.3.3 Genotyping

Genotyping was carried out on 46 samples for which target DNA quantity was estimated to be equal or greater than 0.5 ng (14 summer and 32 winter) and on 26 samples (13 summer and 13 winter) for which nuclear amplification during the screening process failed. Out of the total samples processed and found to be originating from swift fox, 21% (13/61) summer and 40% (30/75) winter-collected faecal samples were successfully profiled to the individual level. Across the nine loci, the average POI was calculated to be 2.55 EX-9. Based on the low POI value, out of the 46 samples sequenced, a minimum of 21 unique genotypes were identified and accepted: 9 genotypes were generated from the summer-collected samples and 15 genotypes from winter-collected samples. Three genotypes from the winter samples were recaptures from the summer survey (Figure 8, Table 8, Appendix 3). In contrast, the 26 problematic samples were unsuccessful at producing reliable profiles and a high proportion of loci were homozygous indicating a high level of allelic dropout (Appendix 4).

During both summer and winter surveys, new individuals were captured on each day (Figure 9) and no individual was recaptured in a different township. A significant difference was found between the total number of foxes captured using the live trapping method and the number generated from the noninvasive winter bait-station method ( $P=0.0422$ ,  $DF=5$ ). However, no significance difference was noted between the two survey methods when data was divided into high and low density townships ( $P > 0.05$ ,  $DF=2$ ) although the sample size was very low.



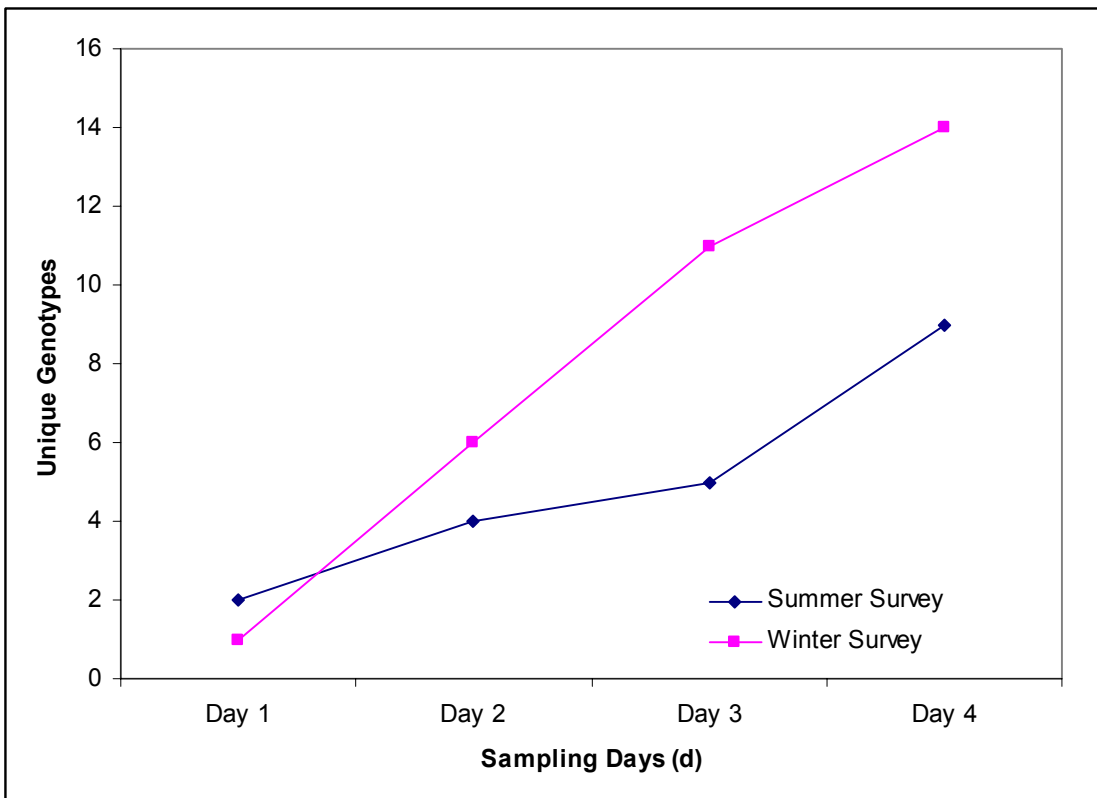


**Figure 8** – Location of unique swift fox genotypes captured at bait-stations during the summer and winter survey. Numbers represent the unique identification of each individual captured at each station.

**Table 8** - Total number of foxes captured in each township during the 2005-2006 Swift Fox population census compared to the total number of accepted unique genotypes generated from profiling nine microsatellite loci. The category “Individuals” represents actual number of animals caught per township during the live-trapping census (Data from Moehrensclager and Moehrensclager 2006).

	Live-trapping Individuals	Bait-station Generated Genotypes		
Township	Winter	Summer	Winter	Total
1. Govenlock	3	3	1	3
2. Nashlyn 1	6	2	4	5
3. Nashlyn 2	7	2	3	4
4. Battlecreek 1	4	2	2	4
5. Battlecreek 2	4	0	4	4
6. Battlecreek 3	1	0	1	1
<b>Total</b>	<b>25</b>	<b>9</b>	<b>15</b>	<b>21</b>

**Figure 9** – Unique genotypes confirmed from each sampling day in summer and winter sampling period in southwestern Saskatchewan.



## 4.4 DISCUSSION

It has been pointed out that fresh faecal samples from small and rare populations can be challenging to locate (Piggott 2004), yet this preliminary study confirms that fresh (< 1 day old) swift fox faecal samples for genetic analysis can be easily collected in summer, fall, and winter seasons in both high and low density areas. Although searching roads and trails resulted in a higher proportion of samples, when the unit effort was taken into account the bait-station method resulted in more faecal samples per day (9.0 - 11.0 samples/day) than the roads/trails method. In contrast, to scent-station studies that report low detection rates of foxes, such as observed in South Dakota (15%) (Uresk et al. 2003) or in California for kit foxes (11%) (Warrick & Harris 2001), the results of this study show high swift fox visitation and scat deposition rate (41-71%) at bait-stations. Although more faecal samples were collected in the summer in low density areas using the roads/trails survey method, the bait-station method produced more faecal samples in both density areas during the winter survey. In addition, the bait-station method was consistent in recovering samples in both high and low density townships across all seasons (Table 7). Most importantly, the baiting method appears to be highly species-specific since only 3% of samples collected in the field were classified as “unknowns” based on morphological characteristics and only 4% of samples chosen for molecular species verification originated from other canid species.

By amplifying two LMW and HMW loci and comparing amplification bands with known amounts of control DNA, target DNA was quantified and samples

with high quality swift fox target DNA were genotyped to the individual level. A greater number of winter-collected samples produced high quality DNA and were carried over for genotyping. Microsatellite profiling and PI estimates confirmed 9 and 15 individuals captured in our study area during the summer and winter sampling periods respectively. A higher proportion of individuals being captured during the winter period was anticipated since this survey period would have included the juveniles which were old enough to utilize the home range. Furthermore, adult swift foxes have larger home ranges during the winter period than during summer (Olson & Lindzey 2002; Kitchen et al. 2005), thus they are more likely to encounter a bait-station and mark it.

The number of unique genotypes generated from the winter survey using the bait-station method was less than the number of animals captured using the live-trapping method which occurred during the same time period. This is surprising since it was expected that the bait-station method would increase the probability of more individuals being captured than the live trapping method. However, it is important to note that a large proportion of samples collected (77% summer and 57% winter) were not selected for genotyping analysis. Thus, the stringent screening method adopted in this study aimed at reducing genotyping errors also resulted in less individuals being genotyped. As Creel et al. (2003) pointed out, the disadvantage of screening samples prior to genotyping is that certain population biases may be introduced if samples are not rejected at random, thus, this approach cannot ensure absolute population estimates. It is acknowledged that further research, such as analysis of poor-quality samples

from areas where unique individuals were not confirmed, would be useful in order to minimize the effect of screening and generate more reliable estimates.

#### **4.4.1 Reliability of Data**

In this study, several steps were taken to decrease genotyping errors and provide reliable genetic data. First, negative controls, samples without DNA, were included in each reaction, and all extracts were prepared in a non-DNA area to prevent contamination from pre-amplified DNA. Secondly, screening of samples through the quantification of target DNA prior to genotyping eliminated a high proportion of samples at risk of high genotyping error as the result of low quantity and quality DNA (Taberlet et al. 1996). To avoid the false classification of one or more individuals as same individual because their genotype is identical, also referred to as the “shadow effect” (Mills et al. 2000), a high number of hypervariable loci with a high level of heterogosity were used to profile swift foxes. Specifically, Morin et al. (2000), recommended genotyping a minimum of seven independent loci such that the PI decreases and genetic errors can be avoided. As well, each locus was amplified separately as multi-locus amplifications have been found to be a source of errors. Lastly, the established haplotype for each sample was compared for each unique individual and no discrepancies were found in the data set.

#### **4.4.2 Conclusion**

In summary, the winter bait-station survey method was effective in recovering fresh swift fox faecal samples for DNA extraction and individual identification. To make the survey more cost-effective driving a truck rather than ATVs is recommend such that six transects could be set up and surveyed in a single day by a single work unit. Based on the field data and the genetic results presented here it is recommended that noninvasive swift fox population surveys be carried out in mid to late winter such that a higher proportion of the population can be captured. For temperate zones where temperatures do not fall below zero, the bait station method can still be carried out successfully to obtain fresh faecal samples for genetic analysis. However, the optimum season that would yield high quality DNA and higher amplification rates would have to be determined prior to a large scale survey. For example, Piggott et al. (2004) reported that spring conditions in Australia were less favorable for red fox faecal genotyping than summer conditions. The lower amplification success and higher genotyping error rates observed for spring-collected samples was attributed to higher levels of precipitation and moisture which is known to degrade the DNA molecule. Overall, faecal DNA genotyping has the potential to become a practical noninvasive survey technique for the Canadian swift fox, providing significant and distinct population data, and overall benefiting future management and conservation efforts. Given that a high proportion of samples were rejected during the screening process, it is highly recommended that more effort needs to

be placed in collecting more samples such that more individuals get captured and identified.

## 4.5 REFERENCES

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## **CHAPTER 5**

### **Recommendations and Conclusion**

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#### **5.1 MANAGEMENT IMPLICATIONS**

The results of this study demonstrate that DNA extracted from swift fox faecal samples for the purpose of species and individual identification is a viable noninvasive approach to study swift fox populations in Canada. In particular, the bait- station technique proved to be an efficient method to collect fresh swift fox samples for molecular analysis. Winter samples produced higher quantity and quality target DNA than summer samples and resulted in more genotypes being generated. Thus, the application of the bait-station technique for collection of faecal samples followed by molecular analysis has the potential to study the spatial distribution of swift fox populations (ie. presence/absence) as well as estimate population size.

##### **5.1.1 Geographic Distribution**

A major priority outlined in the *Recovery Strategy for the Swift Fox* is to “identify and initiate the securement of swift fox habitat necessary to achieve recovery goals” (Pruss et al. in review). Currently, 75% of swift fox occupancy range is being sampled through the live-trapping population census (Moehrensclager & Moehrensclager 2006). As such, noninvasive sampling through species presence/absence surveys has great potential for identifying

swift fox habitat and determining geographic distribution in areas previously not sampled or currently unknown to be occupied by swift fox.

This study has demonstrated that swift fox faecal samples can be easily obtained in the wild from bait-stations but cannot be correctly identified based exclusively on morphological characteristics. Thus, species identification through mtDNA control region amplification and sequencing can be carried out to accurately determine the species and the overall spatial distribution. As observed in Chapter 3, mtDNA amplification success was obtained from a high proportion (92%) of selected samples indicating that species identification is effective and a viable option for estimating relative abundance. Furthermore, there was no significant difference of amplification success among the two seasons suggesting that sample collection for the purpose of species identification could be carried out in both summer and winter conditions. However, based on biological factors, swift fox surveys are recommended to be carried out in mid or late winter when more individuals are present on the landscape (*see Survey Timing below*).

### **5.1.2 Estimating Population Size**

Globally, there is a huge demand to adopt less invasive survey techniques to estimate population size, specifically for rare and endangered species, and new models are continuously being developed and tested (Wilson et al. 2003; Frantz et al. 2004; McKelvey & Schwartz 2004; Bellemain et al. 2005; Lukacs & Burnham 2005; Miller et al. 2005). However, several factors must be considered

before an effective survey design and population estimation model are adopted. First, for mark-recapture models such as the Lincoln-Petersen estimator, one underlying assumption is that tags (ie. genotypes) are not lost or overlooked by the investigator (Lettink & Armstrong 2003). In noninvasive genetic sampling, the acceptance of an incorrect genotype due to genotyping error can have serious implications on the estimator's assumptions and on the overall population estimate. In this study, genotyping error was neither quantified nor minimized using the multitubes approach, thus it cannot be alleged that the genetic data are error free. Furthermore, another important assumption of mark-recapture models is that capture probabilities are equal among sampling periods (Lettink & Armstrong 2003). Software packages such as MARK, CAPTURE, and *capwire* have been developed to deal with capture heterogeneity encountered in natural populations (Waits 2004; Miller et al. 2005). The preliminary results of this study suggest that DNA extracted from faecal samples collected in winter has the potential to become a valuable tool for individual identification and population size estimation if recapture probability is increased through collection of more faecal samples.

## **5.2 SURVEY DESIGN RECOMMENDATIONS**

The following themes are provided as a guide in developing an effective noninvasive survey protocol for determining both swift fox spatial distribution and population size.

### **5.2.1 Survey Timing**

The timing when a population survey is carried out is critical in the establishment of reliable and robust population parameters. Most scent-station studies recommend late summer or fall as the optimum seasons for conducting swift fox surveys since the likelihood of detecting an individual is increased by the greater mobility and dispersal of juveniles and adults during this period (Uresk et al. 2003). Although high visitation rates are desirable, for conservation and management purposes it has been suggested that spring estimates, which reflect the size of the breeding population, is more important than fall estimates which represent dispersing juveniles (Lettink & Armstrong 2003; Sargeant et al. 2003). Based on the results of this study, it is recommended that faecal DNA swift fox population surveys be carried out mid or late winter for several reasons. Field data using the bait-station method showed higher sample recovery and scat deposition rates during the winter season than during late summer. This could be attributed to several ecological factors. First, during the summer sampling period juveniles were not captured since they are close to the natal den and hunt in the vicinity given that prey species are abundant during this season. Additionally, adult swift fox have smaller home ranges during the summer period when they



are rearing their pups (Olson & Lindzey 2002; Kitchen et al. 2005), thus a smaller area is being searched and less individuals captured. Furthermore, swift fox activity patterns, meaning the distance traveled per hour, is higher during the breeding season than during the summer (Kitchen et al. 1999). In Canada, breeding season starts around February 15 (Moehrenschrager & Macdonald 2003), thus surveys could be started after this date in order to increase probability of foxes encountering and marking bait-stations. One key component of the survey is that nuclear amplification success and target DNA quantity and quality from swift fox faecal samples were found to be significantly higher for winter-collected than summer-collected samples (see *Chapter 3*). Therefore, the greater number of recovered faecal samples coupled with higher target DNA amplification success resulted in a greater number of individuals being identified from the winter-collected samples.

Finally, when a survey method is being developed, it is worth considering logistical issues and ease of access to the area given that a high portion of swift fox habitat falls within private and federal property. For example, the presence of cattle in all the townships surveyed during the summer period posed a challenge since the PFRA land managers were opposed to motorized vehicles being driven in the pastures when cattle were present. Similarly, landowners are reluctant to grant access to pastures with grazing cattle for fear of gates being left open and cattle mixing with other cattle or ending up on roads. Another serious issue encountered during the summer season is fire hazards. Summer conditions are characteristically hot with prolonged periods of little or no precipitation. Both

landowners and PFRA managers are opposed to having vehicles on pastures even when they are exclusively driven on designated trails. This is primarily because most pasture trails have a narrow ribbon of grass which can accumulate underneath the vehicle and ignite from the hot motor.

Therefore, based on the results of this study, winter-surveys are recommended. However, it is important to note that winter surveys would be challenging given that local weather is very unpredictable; blizzards and snow accumulation in low elevation areas can hinder road and transect access. Under these circumstances the use of snowmobiles is recommended.

### **5.2.2 Sampling Frequency**

Based on the field and genotyping data it was concluded that four operation days were not sufficient in capturing a high proportion of individuals, thus a greater number of sampling days is recommended to capture more individuals and to minimize unpredictable weather effects. For example, a low number of faecal samples were recovered after a very windy day and night probably as a result of the scent of the bait being dispersed and ineffective in attracting foxes. Additionally, the genotyping data indicated that new individuals were captured on each new sampling day and that an asymptote was not reached even after the four sampling days. Therefore, if population size would be estimated using the asymptote approach (Kohn et al. 1999), more than five days would be required to capture a high proportion of individuals in the study area. Another way to increase the proportion of the population being captured using

noninvasive methods is by collecting more samples. To increase sample size without increasing sampling days, it is recommended that transects be revisited at three day intervals rather than each consecutive day.

### **5.2.3 Bait-station Design & Spacing**

The benefit of the bait-station, which consists of a wooden stake wedged in the ground and baited with sardines, is that it is inexpensive, easy to set up and take down, and requires little training of field personnel. However, it is important to note that this design might not be ideal in all circumstances. For instance, during the 2006 fall survey, after three days of visits only two faecal samples were collected in Govenlock even though the bait was continuously removed and obvious fox tracks in the snow were observed around the bait-stations. Govenlock was considered a high swift fox density township, where seven foxes were caught two weeks prior to the bait-station fall survey (Moehrensclager, unpublished data), thus the low number of faecal samples collected was questionable. According to Sargeant et al. (2003) swift foxes can become habituated to scent-stations baited with odour attractants after the first night causing visitation rates to decrease. The habituated behaviour observed only in this particular township could be attributed to the fact that wood stakes identical to the ones used for the bait-station design in this study have been extensively used by the oil and gas industry to mark roads and pipe line construction. Because in our study area the bait consisted of sardines that the

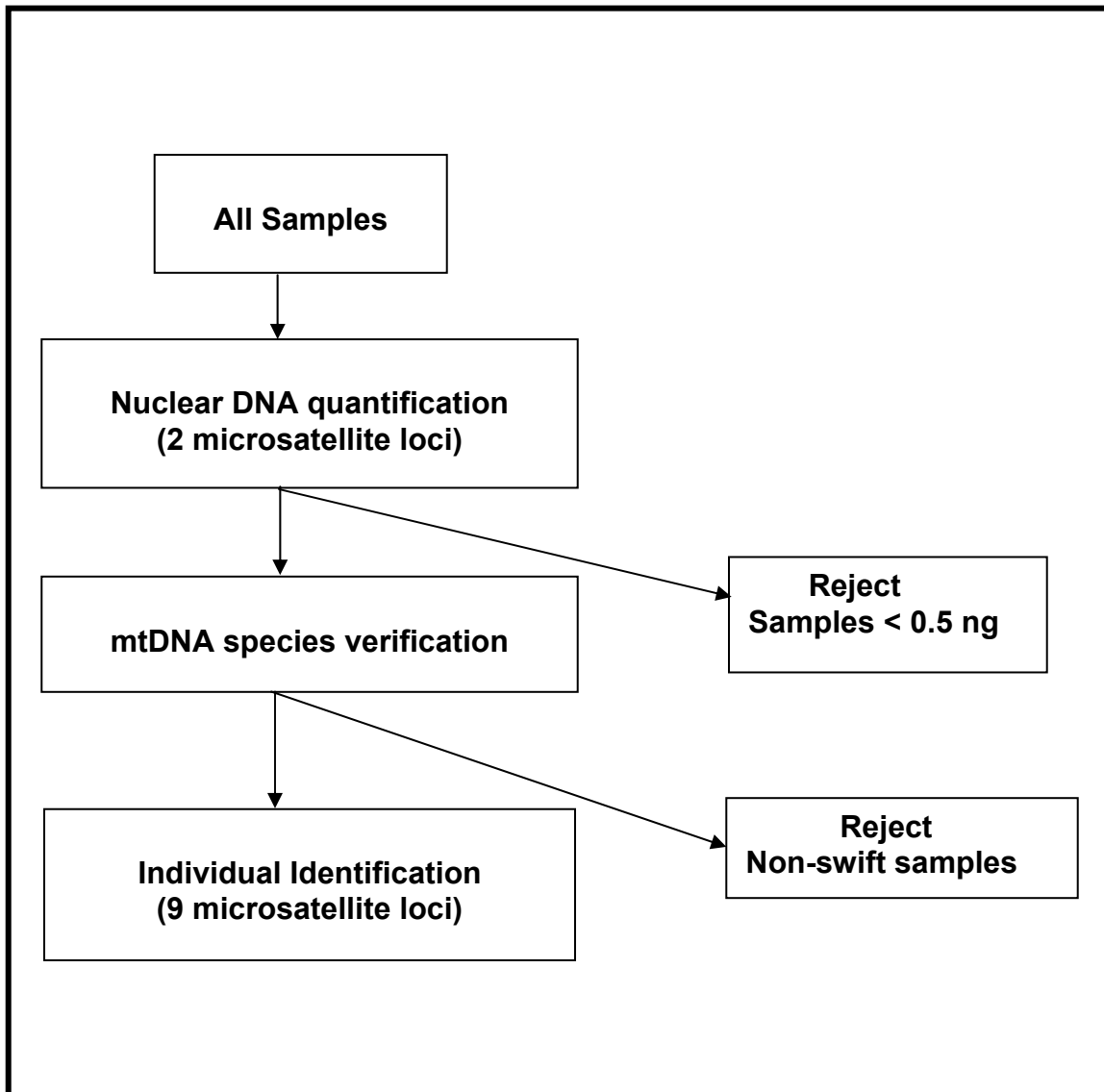
foxes could consume, it is possible that the foxes continued to visit the stations for the bait but not longer perceived the bait-station as a new and interesting object that necessitated to be marked for future references. As oil and gas activities are expected to continue and expand in swift fox habitat, the habituated response of foxes to wood stakes requires further examination.

For fox scent-station surveys, the placement of closely spaced stations has been commonly recommended to increase sampling effort in low density areas (Uresek et al. 2003). However, in this study, the 5-km transect in the middle of the township with bait-stations placed at 0.5 km is believed to have been effective in attracting swift foxes in both high and low density areas. That is, no significant difference was observed between the number of individuals in both the low and high density areas estimated through the generated genotypes from the winter survey and the number of individuals captured during the live-trapping census. However, to reduce the costs and efforts which are associated with systematic sampling, stratification of the study area into high-density and low-density areas is highly recommended for future surveys.

#### **5.2.4 Sample Screening**

It has been stressed that the only way to minimize genotyping errors in a data set is to repeat typing each extract at each locus until a consensus is reached (Taberlet et al. 1996; Creel et al. 2003). For a large scale population study utilizing a multi-locus genotype tag, this method can become expensive and time consuming considering that the quantity of target DNA available for

PCR is not even known prior to genotyping (Ball et al. 2007). The approach of this study was to quantify target DNA using two microsatellite loci prior to nuclear amplification and to use only the samples with high quality target DNA (> 0.5 ng) that can produce successful profiles in a single round of amplification (Figure 10). As seen in this study, problematic samples were unsuccessful at being profiled and produced a high proportion of homozygous loci (Appendix 4). Thus, it is highly recommended that samples with high quantity and quality target DNA are identified at an early stage prior to sequencing, thus minimizing genotyping errors, and maximizing efficiency of laboratory effort and cost. Some studies have adopted the amplification of mtDNA as an initial screening step prior to sequencing. However, this approach is not recommended for swift fox faecal samples since mtDNA amplification success was very high in comparison to the nuclear DNA amplification during the screening phase. As such, rejecting a high proportion of samples that do not provide high quality nuclear DNA and cannot be used for individual identification can be effective in cutting down costs on species identification analysis.



**Figure 10** - Screening sequence recommended for analyzing swift fox faecal samples.

### **5.3 FUTURE RESEARCH**

Since this pilot project was the first to evaluate the possibility of faecal DNA genotyping as a population survey technique for the Canadian swift fox, the need for future research is immense. As such, it is recommended that testing this novel technique be continued and that laboratory methods be further optimized. The following are some issues that require further investigation if this technique is to be applied in the future at the range scale.

1. Swift fox microsatellite primers must be further optimized (C. Cullingham, pers. comm., 2007).
2. Genotyping error rates must be quantified.
3. Markers for sex determination must be tested.
4. A population survey at a larger scale using the bait –station method to obtain faecal samples must be conducted and the capture probabilities in high and low density areas must be determined.

### **5.4 CONCLUSION**

To date, live-trapping has been used as the conventional method to assess swift fox population levels and geographic distribution despite the species' endangered status in Canada. Although live-trapping of individuals provides valuable population data, trapping is intrusive and poses risk of injury and distress to the animals. There is a growing concern to reduce risk of injuries

of trapped animals (Moehrenschrager et al. 2003) and to develop noninvasive methods to study endangered and wild populations (Ernest et al. 2000; Smith et al. 2005; Sugimoto et al. 2006). Effective survey techniques to monitor trends in population abundance and spatial distribution are critical for the successful conservation of the Canadian swift fox.

Noninvasive genetic sampling has become a powerful research tool with a wide range of applications for conservation biology and wildlife management and as species-specific markers become developed and more available, faecal DNA sampling will become a cost-effective approach for studying many species. In particular, faecal DNA sampling using the bait-station method has the potential to be applied at the large landscape scale and provide valuable population data for effective management applications. For example, under ideal weather conditions, a single work unit (ie. two people per vehicle) could survey the 237 townships that were surveyed during the 2005-2006 Swift Fox Population Census in approximately 40 days (not including time off and traveling time), completing the census in less time and men power required for the live-trapping population census. In the broader conservation context, the technique can be applied to obtain other important population parameters including density, spatial and temporal distribution, sex composition, dispersal and genetic exchange patterns as well as phylogenetic relationships and genetic variation. Presence of predators such as coyotes and red foxes can be deduced from collecting faecal samples along roads and trails and identifying them to species. In conclusion, collecting fresh swift fox swift faecal samples using bait-stations followed by



species identification and microsatellite genotyping has the potential to be used for population estimates and geographic distribution in a noninvasive fashion, avoiding the need to live trap individuals and contributing to this species recovery on the Canadian prairies.

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## APPENDIX 1

### Data Collected in the Field

**GPS Location**

**Date & Time**

**Sample Number**

**Surveyor's Name**

1. Medea Curteanu
2. Maureen Hodgins
3. Daniel Smith
4. Jennifer Lusk

**Survey Method**

1. Roads/Trails
2. Baited
3. Opportunistic

**Township**

1. Govenlock
2. Nashlyn 1
3. Nashlyn 2
4. Battlecreek 1
5. Battlecreek 2
6. Battlecreek 3

**Habitat**

1. Native Grass – grazed
2. Native Grass – ungrazed
3. Agricultural
4. Planted Grass
5. Planted & Native Grass

**Location**

1. Unpaved road
2. Trail
3. Grass Trail
4. Bait Station
5. Road intersection

**Scat Diameter**

1. Diameter >20 mm – coyote
2. Diameter < 20 mm – fox
3. Diameter < 20 mm – unknown

**Scat Age**

1. < 1 day
2. < 2 days
3. < 3 days
4. Unknown

**Scat Freshness**

1. Very Fresh
2. Fresh
3. Dry
4. Old

**Number of Scats**

## APPENDIX 2

### Capture History

Capture history of nine swift fox individuals identified from the summer survey. Townships are represented by letter “T” while bait-stations are represented by letter “B”.

Individual ID	t <sub>1</sub>	t <sub>2</sub>	t <sub>3</sub>	t <sub>4</sub>	Total Captures	Recaptures in winter
1	1	0	0	0	1	
	T4_B6					
2	1	0	0	0	1	
	T4_B8					
3	0	1	0	1	2	1X
		T3_B2		T3_B1		
5	0	1	0	0	1	
		T1_B10				
6	1	0	0	0	1	2X
	T1_B5					
7	0	1	0	0	1	
		T1_B7				
9	0	0	0	1	1	
				T2_B11		
10	0	0	0	1	1	
				T3_B4		
16	0	1	0	0	1	2X
		T2_B9				

Capture history of 14 swift fox individuals identified from the winter survey. Townships are represented by letter “T” while bait-stations are represented by letter “B”.

Individual ID	t <sub>1</sub>	t <sub>2</sub>	t <sub>3</sub>	t <sub>4</sub>	Total Captures
3	0	0	1	0	1
			T3_B1		
4	0	0	1	0	1
			T6_B4		
6	0	1	0	1	2
		T1_B5		T1_B2	
8	0	1	0	1	2
		T2_B7		T2_B10	
12	0	0	0	1	1
				T3_B5	
13	0	1	0	0	1
		T5_B3			
14	0	0	1	0	1
			T2_B2		
15	0	0	0	1	1
				T2_B2	
16	0	0	1	1	2
			T2_B4	T2_B3	
17	0	0	0	1	1
				T5_B8	
18	0	0	0	1	1
				T5_B9	
19	0	1	0	0	1
		T4_B2			
20	0	0	1	1	2
			T6_B5	T6_B5	
21	0	0	0	1	1
				T3_B11	

## APPENDIX 3

Swift fox genetic profiles based on nine microsatellite loci. The first column indicates whether the sample represents a unique individual and samples representing the same individual are grouped together. Samples marked with a question mark indicate samples for which a unique identification was not determined. Missing data is represented by '\_'. (Results provided by C. Cullingham).

INDIV	SAMPLE	VVE3131	VVE3131	VVE533	VVE533	VVE545	VVE545	VVE264	VVE264	VVEM19	VVEM19	VVEM17	VVEM17	VVEM25	VVEM25	VVE2111	VVE2111	VVE2110	VVE2110
1	1188	188	192	260	260	267	275	230	230	391	395	419	423	397	397	354	362	270	270
2	1201	192	196	260	260	271	275	218	226	387	391	419	419	397	419	354	362	267	270
3	1207	164	188	252	264	279	279	230	230	404	408	419	423	443	443	354	362	267	270
	1266	—	—	—	—	—	—	230	230	404	408	—	—	443	443	—	—	267	270
	1628	—	—	—	—	—	—	230	230	404	408	419	423	—	—	354	362	267	270
4	1432	192	196	260	260	271	279	218	234	391	396	415	419	—	—	354	358	270	270
5	1248	192	192	244	256	275	279	218	230	408	408	423	427	429	443	358	362	270	357
6	1249	192	196	256	268	265	275	230	230	408	408	415	423	397	429	354	358	267	300
	1252	—	—	256	268	—	—	230	230	408	408	415	423*	397	429	354	358	267	300*
	1413	192	196	256	268	265	275	230	230	408	408	415	423	397	429	354	358	267	300
	1492	192	196	256	268	265	275	230	230	—	—	415	423	397	429	354	358	—	—
7	1258	192	192	244	256	275	275	218	230	387	408	423	427	—	—	358	362	270	270
	1259	—	—	244	256	—	—	218	230	387	408	423	427	429	443	358	362	270	270
8	1433	—	—	256	264	—	—	218	230	387	408	415	419	408	429	362	362	270	305
	1437	188	192	256	264	267	279	218	230	387	408	415	419	408	429	362	362	270	305
	1509	—	—	256	264	267	279	218	230	387	408	419	423	408	429	362	362	270	305
9	1264	192	196	256	260	275	275	226	230	390	408	415	423	—	—	354	362	267	293
10	1267	—	—	260	264	—	—	226	230	387	390	415	419	405	425	354	362	267	293
	1268	192	196	260	264	275	279	226	230	—	—	415	419*	405	425	354	362	267	293
11	1390	—	—	264	268	275	279	—	—	386	408	415	423	—	—	—	—	267	267
12	1391	—	—	—	—	—	—	218	230	379	408	423	427	419	443	—	—	267	305
	1392	164	192	252	260	271	275	218	230	379	408	423	427	419	443	354	362	267	305
13	1441	188	192	256	268	271	279	214	230	395	408	415	423	431	443	354	362	296	300
	1446	—	—	256	268	271	279	214	230	395	408	415	423	431	443	354	362	296	300
14	1444	188	192	244	256	267	275	218	230	379	408	419	423	429	443	362	362	305	363
15	1496	188	192	244	260	269	271	230	230	395	408	419	423	443	443	354	354	—	—
16	1212	—	—	244	260	279	279	—	—	—	—	415	423	429	429	362	362	300	357
	1480	—	—	244	260	279	279	218	226	—	—	—	—	429	429	362	362	300	357
	1481	188	192	244	260	279	279	218	226	—	—	415	423	—	—	362	362	300	357
	1482	188	192	244	260	279	279	—	—	383	391	415	423	429	429	362	362	300	357
	1497	—	—	244	260	—	—	218	226	383	391	415	423	429	429	362	362	300	357
	1498	—	—	244	260	279	279	218	226	383	391	415	423	429	429	362	362	300	357
	1500	—	—	—	—	—	—	218	226	383	391	415	423	429	429	—	—	300	357
	1505	188	192	244	260	279	279	218	226	383	391	415	423	429	429	362	362	300	357

INDIV	SAMPLE	VVE3131	VVE3131	VVE533	VVE533	VVE545	VVE545	VVE264	VVE264	VVEM19	VVEM19	VVEM17	VVEM17	VVEM25	VVEM25	VVE2111	VVE2111	VVE2110	VVE2110
16	1507	—	—	244	260	279	279	218	226	383	391	415	423	—	—	362	362	—	—
17	1613	188	196	260	268	271	271	230	234	391	395	415	419	443	464	354	358	270	296
18	1616	192	196	256	264	267	275	222	230	—	—	—	—	—	—	362	362	300	363
19	1623	—	—	—	—	—	—	230	230	390	395	—	—	431	443	—	—	270	305
	1624	164	192	260	260	271	279	230	230	390	395	415	423	431	443	354	358	270	305
20	1626	164	188	256	268	279	279	226	230	391	408	419	423	429	443	354	362	300	305
	1627	—	—	256	268	—	—	226	230	391	408	419	423	429	443	354	362	300	305
	1631	164	188	256	268	279	279	226	230	391	408	419	423	—	—	354	362	—	—
21	1389	164	164	260	260	271	279	230	230	—	—	415	423	—	—	354	358	270	270
?	1499	—	—	—	—	—	—	218	226	383	391	—	—	—	—	—	—	300	357
?	1221	—	—	—	—	—	—	218	234	—	—	—	—	—	—	—	—	270	270
?	1508	—	—	—	—	—	—	218	230	—	—	—	—	—	—	—	—	—	—



## APPENDIX 4

Swift fox genetic profiles for the 26 problematic samples. Homozygous loci are highlighted in yellow.  
(Results provided by C. Cullingham 2007).

Sample	VVE2-64	VVE2-64	VVE5-33	VVE5-33	VVE-M19	VVE-M19	VVE-M25	VVE-M25	VVE2-110	VVE2-110
1035	218	218							308	308
1193	-	-								
1195	230	230					372	372		
1213	-	-								
1230	-	-								
1241	-	-								
1244	230	230								
1250	218	226								
1263	-	-								
1265	-	-								
1272	-	-								
1276	218	226			390	390			258	315
1393	230	230			385	406				
1426	218	218	256	256	406	406			305	305
1434	218	226	244	260	383	383			300	300
1435	226	226			390	390	429	429	357	357
1445	218	226			406	406			363	363
1484	-	-								
1485	214	218			383	390			300	300
1502	-	-								
1614	-	-								
1622	230	230	264	264	408	408				
1625	230	230								
1239	-	-								
1495	230	230								