Relatedness and the effect of inbreeding on mortality in Sable Island horses

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Abstract

It is often difficult to asses the social and mating structure of populations through the usage of observational methods alone. Molecular methods give greater insight into patterns of relatedness within populations, and with the recent application of network theory, allows for the quantification of those patterns of relatedness. This can be used to examine aspects of population such as mating patterns, reproductive success, dispersal and inbreeding. Small populations will lose genetic diversity faster than larger populations through genetic drift and inbreeding, and this genetic decline can lead to issues with fitness. Sable Island is a small island located off the eastern coast of Nova Scotia, which contains a small population of feral horses. Little is known about their social structure, or the factors behind the fluctuations of population size that occasionally occur. This study aimed to assess their natal dispersal patterns and reproductive success, as well as to assess the degree to which inbreeding affects mortality in harsh winters. DNA was extracted from samples collected from 208 individuals that died in a large mortality event in 2003. Genotype data from these individuals were used to estimate relatedness. A network was generated, and used to test for correlation between relatedness patterns and habitat use patterns, and to test the reproductive success of males and females. No correlation between groups of related individuals and location in females was seen, indicating that females are not remaining in natal areas as expected. A negative correlation between the two was seen in males, indicating that they are likely avoiding areas with close kin. Males were also had overall lower reproductive success, and more variation in reproductive success than females, which is expected of a polygynous mating system. No connection between inbreeding and mortality was seen, as levels of inbreeding were similar across all age classes.

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Introduction

The use of genetic analyses alongside traditional field methods when studying reproductive strategies and social structure has become common. Using both methods gives greater insight into mating systems than either alone, as using only observational field methods may not accurately depict the situation either due to difficulties in observation or unobservable factors (Coltman *et al.* 1999a); and the field data provides the context within which the genetic data can be interpreted. This combination has led to a better understanding of reproductive success in animals and greater accuracy when assessing mating structure and patterns of relatedness (Hughes 1998), and has been expanded upon in recent times by the application of network theory to social and genetic data as a way to visualize and quantify relationships within populations (Croft *et al.* 2008). These techniques can be used to examine aspects of a population such as mating structure, reproductive success, inbreeding, dispersal and interaction with kin (Hughes 1998).

Field and Molecular Data

While observational studies may provide an accurate assessment of relatedness in some species it is not sufficient to fully explain the mating systems of others. In Soay sheep, *Ovis aries,* less than a third of those males seen in consort with an ewe were found to be the sire of her offspring when paternity was tested through molecular means. Although those males seen in consort were still significantly more likely to be the sire than a randomly chosen male it illustrates the deficiencies in relying on observations

alone (Coltman *et al.* 1999a). This is seen in other species such as seals (Worthington Wilmer 1999; Coltman *et al.* 1998), for example, as some of their mating activity occurs under water. Observing copulations that occur outside of consorts can often be difficult except by means of continual observation of an individual and even then if a female were to mate multiple times no conclusive determination of paternity can be reached. Extra-pair matings occur as there are benefits to infidelity for females in the form of increased fitness of their offspring, particularly if the male she is paired with is genetically similar due to social restrictions on mate choice (Blomqvist 2002) or is of relatively poor quality (Petrie and Kempenaers 1998; Brown 1997). Where continual observation of individuals is often not feasible or even possible the use of molecular means becomes essential.

The study of Soay sheep also exemplifies the importance of sperm competition as a factor in reproductive success. In addition to the relative dominance of the male, testes size and number of sperm also influence the number of offspring sired in Soay sheep (Preston *et al.* 2003). The importance of sperm competition was first examined in insect models and occurs when multiple males are mating with the same female and thus multiple sperm competing for the same ova (Parker 1970). Those species that exhibit promiscuity put more energy into sperm competition than those who remain monogamous due to the increased likelihood of multiple males mating with the same female (Simmons & Fitzpatrick 2012), resulting in traits such as larger testis and thus more sperm-producing seminiferous tissue (Preston *et al.* 2003; Lüpold *et al.* 2009). This morphological adaptation, as well as other changes to the male reproductive system and reproductive strategies seen among those animals that are subject to sperm competition, are present not only in the Soay sheep but a variety of animals. These include the house

mice (*Mus musculus*) (Firman & Simmons 2008), fruit flies (*Drosophila melanogaster*) (Moatt *et al.* 2014), Monarch butterflies (*Danaus plexippus*) (Solensky and Oberhauser 2009), fish (Smith *et al.* 2009) and primates, including humans (Harcourt *et al.* 1981). Post-copulatory mechanisms of sexual selection and their success cannot be measured through behavioral observations as they occur within the female's reproductive tract.

Recently network analysis has been applied to the examination of complex social structures and patterns of relatedness. Network analysis originated in mathematics in the form of network theory however it has been applied to a growing number of fields (Wey *et al.* 2008). When examining animal populations each individual is considered a "node"; and their connections with other individuals, whether it be social or genetic in nature, defined as "edges". Through the use of network analysis and the examination of edges between individuals, groups of connected individuals may be identified, and individual levels of connectedness can be assessed (Wey *et al.* 2008). This application of network theory can provide insight into interactions between individuals and aspects of social structure, as well as providing the capability to visualize and quantify the occurrence and strength of relationships through the creation and analysis of networks (Croft *et al.* 2008). Aspects of both individuals and entire groups can be measured, such as the number and strength of relationships an individual has to others, or how closely connected a group of individuals is (Wey *et al.* 2008). It is being used with increasing frequency to gain insight into the social structure of populations through studying interactions between individuals (Krause *et al.* 2007), and may be applied in similar ways to genetic information in order to analyze patterns of relatedness.

The examination of patterns of relatedness has proven to be a useful tool in the assessment of social structure. Looking at relatedness can reveal complexities in aspects of group structure such as mating habits, inbreeding, dispersal and interaction with kin (Hughes 1998). Genetic analysis of paternity and relatedness can be used both to examine reproductive success of an individual, and to give insight into the population's mating system through the comparison of individuals. In populations such as humpback whales (*Megaptera novaengliae*), the hypothesis that they follow a polygynous mating system can be tested in part through the examination of variance of reproductive success between males, which suggested a nonrandom mating system with some males at a slight advantage (Cerchio *et al.* 2005). Similarly, paternity analysis in North Atlantic right whales (*Eubalaena glacialis*) showed differential reproductive success among males, and supported the idea that intense sperm competition is occurring within the population (Frasier *et al.* 2007). Analysis of patterns of relatedness in the brown long-eared bat (*Plecotus auritus*) showed little variance in reproductive success between males, however the presence of full siblings within the population indicated that these bats sometimes show loyalty to mating sites or males between years (Burland *et al.* 2001) as they are having multiple offspring by the same male. These studies highlight the usefulness of the analysis of patterns of relatedness in revealing details about the mating systems of populations.

Patterns of relatedness may also be used to examine group membership and patterns of natal dispersal. This can be done through comparison of relatedness between individuals to observational information on location and group membership. If clusters of related individuals correlate with location or group membership, then individuals are

5

staying near relatives and not dispersing from natal areas; conversely low correlation between the two would suggest that individuals are dispersing. A study by Miyamoto *et al.* (2013) on dispersal in red colobus monkeys (*Procolobus rufomitratus*), which has proven difficult to quantify through observation in this species, combined the use of microsatellite data with field data on characteristics of groups, such as membership and size. This comparison found that females had higher levels of dispersal than males, and that dispersal levels differed based on group size as larger groups had lower correlation between group membership and clusters of relatedness. Analysis of relatedness within groups of spider monkeys (*Ateles belzebuth*) showed that male relatedness within one of the groups studied was significantly higher than that of females, showing male philopatry. However, no variance between sexes was seen in another group within the same study, showing differences in dispersal habits within the population (Di Fiore *et al.* 2009). Studies such as these highlight the usefulness of patterns in relatedness when examining aspects of social structure, giving insight into aspects that are often difficult to observe otherwise.

Inbreeding

A decrease in heterozygosity within a population can have implications for the health and survival of the individuals within it. In some species females preferentially chose unrelated males as mates (Linklater and Cameron 2009), or exhibit promiscuity in order to find higher quality or less related males. The offspring of females exhibiting higher levels of promiscuity often have better survival rates, disease resistance and growth rates (Brown 1997; Tregenza and Wedell 2002). They also avoid some of the negative effects of inbreeding in cases where one of the males bred with were closely

related, likely due to differential fertilization in favor of genetically dissimilar sperm (Tregenza and Wedell 2002; Gasparini and Pilastro 2011). Avoiding inbreeding increases fitness in offspring as it increases heterozygosity and avoids the expression of deleterious recessive alleles, one of the major causes of inbreeding depression (Charlesworth and Charlesworth 1999). This can have negative consequences such as increased susceptibility to disease, pathogens and parasites (Acevedo-Whitehouse *et al.* 2003; Coltman *et al.* 1999b), as well as to environmental conditions (Liao *et al.* 2009).

A number of factors can have a negative influence on survival during winter months. These may include starvation due to increased difficulty foraging due to snow accumulation or lowered food availability (Oja *et al.* 2013), predation (Adams *et al.* 1995), and high population density (Sinervo *et al.* 2000). The effects of inbreeding depression have also been shown to increase during times of increased environmental stress, such as harsh winters (Liao *et al.* 2009; Cheptou *et al.* 2010), potentially leading to lowered survival rates among individuals showing high levels of inbreeding. This makes small populations particularly vulnerable to severe weather conditions as they are more likely to show a decrease in heterozygosity (Charlesworth and Charlesworth 1999). Understanding the interaction between environmental conditions and factors such as inbreeding, and how this interaction affects survival and risk of extinction, has important implications in terms of conservation (Cheptou *et al.* 2010) and the development of effective management programs.

Horses

There are seven extant equids of which horses exist as one of two subspecies of

Equus ferus; Equus ferus przewalskii and *Equus ferus caballus,* the domestic horse. There are two categories of social system within the genus; one being those that form no permanent associations, with males that defend a territory in which they have exclusive mating rights. Subordinate males in this system compete for possession of territory as it is a requirement to gain access to females. Mares form unstable herds and do not form associations with the males she breeds with. The only stable unit is a mother and her offspring. The equids that follow this system include the African wild ass, *Equus africanus;* Greyv's zebra, *Equus grevyi*; and onager, *Equus hemionus* (Klingel, 1975). The other social system involves the formation of cohorts that last multiple breeding seasons, usually in the form of polygynous groups referred to as harems. Males in this system are non territorial and they maintain their position in harems across breeding seasons; those who do not gain harems often form bachelor bands or exist as solitary individuals. Equids that display this social structure include the mountain zebra, *Equus zebra*; plains zebra, *Equus quagga*; Przewalski's horse, *Equus ferus przewalskii***;** and the domestic horse, *Equus ferus caballus* (Klingel, 1975).

Although often considered in a domestic context, horses once existed as wild animals across North America and Europe and now exist as both domestic and feral populations. Horses are polygynous animals that form stable bands with one or two band stallions and a harem of one or more mares, forming the basis of their reproductive unit (McCort 1977). Changes in membership are often a result of the dispersal of juvenile horses from their natal bands rather than adult horses changing bands (Feist and McCullough 1975). Mares may be aggressive towards juveniles of both sexes, particularly if the mother of the juvenile was not of high status within the band,

pressuring them to disperse (Rutberg and Keiper 1993). Reasons for foal dispersal differ between the sexes, however, and they differentially receive aggression from other band members.

Female dispersal is associated with their first oestrus rather than aggression by residents of their natal band (Rutberg and Keiper 1993; Linklater and Cameron 2009). They often experience little aggression from other members prior to their dispersal, except from band stallions. Females who disperse are often quickly accepted into a band (Monard *et al.* 1996). After becoming integrated into a band, their reproductive success often depends on their age and ability to maintain social relationships (Cameron *et al.* 2009). Their dispersal may be a mechanism of inbreeding avoidance as it is associated with female oestrus and they preferentially chose bands with unfamiliar stallions (Linklater and Cameron 2009). As well in cases where females fail to disperse their fecundity is often impaired (Rutberg and Keiper 1993), possibly as a result of remaining in bands with related males. Females often preferentially disperse to nearby bands (Linklater and Cameron 2009), which often follow similar movement patterns and ranges despite a lack of interaction between them (McCort 1982).

Juvenile males, on the other hand, receive aggression from both males and females in attempts to remove them from their natal bands (Monard *et al.* 1996). Subordinate males represent competition for the band stallion and may sexually harass females within the group, which may influence their health and reproductive success (Linklater *et al.* 1999). This results in an increase in pressure for them to disperse as compared to females. Male dispersal is common in mammals, particularly in polygynous species, and is likely a

9

result of both mate competition and inbreeding avoidance (Dobson 1982). Unlike

females, not all males become a part of bands; those males that do not often form less

stable bachelor groups after either dispersing from their family group or losing their

position as band stallion. Others simply exist as solitary animals in the case of some older

males. Band stallions may be ousted from their position by another male (McCort 1977)

as bachelor males will attempt to steal females or entire harems from band stallions in an

attempt to increase their reproductive success (Feist and McCullough 1975).

Most males may not successfully reproduce during their lifetimes except by means

of sneak copulations. This is due to the unequal sex ratios inherent in a polygynous group;

in most cases only one male will have reproductive access to the females in a band

(Klingel 1975). As well the relatively stable composition of horse bands makes it difficult

for bachelor males to become band stallions, leading to unequal access to females.

Accessing mares from another stallion's harem may prove a difficult task as many

attempts result in aggressive encounters between males, ranging from threat displays to

actual fighting (Feist and McCullough 1975). Interaction between a stallion's harem and a

bachelor band will often result in an aggressive response from the band stallion, however

actual fighting usually results from the approach of a solitary male (Feist and

McCullough 1975; McCort 1977). This aggressive defense of the harem by the stallion

may discourage infidelity by driving off outside males and making it difficult for

bachelors to reproduce.

Paternity analyses on populations of feral horses have found that mares have a

relatively high fidelity to the band stallions. A paternity analysis using blood typing on the

feral horses of Misaki, Japan found that 85% (n=99) of foals born were the offspring of the band stallion (Kaseda and Khalil 1996). An analysis of Great Basin feral horses, also using blood typing, found that 70% (n=80, excluding those who had recently been disturbed by human activity) of foals born were the offspring of the stallions in their band (Bowling and Touchberry 1990). Paternity analysis using blood type has since been replaced with more accurate means such as microsatellite genotyping. Gray *et al.* (2012), using DNA analysis, had found that only 57% (n=51) of offspring in a band belonged to the band stallion. However, this may be the result of human interference as the study was preceded by a roundup of all the horses studied and it is unknown whether the horses returned to the bands from which they originated. The next year of study 5/6 offspring were that of the band stallion, suggesting that the roundup may have been a factor in the apparent low fidelity during the first year of study.

There has been speculation that male aggression and infanticide involving unrelated foals could play a role in the often high fidelity seen in horses. Infanticide has been reported in all extant equids including horses (Gray et al. 2012). It benefits a stallion to commit infanticide if the current foals in a band are not likely his, and if doing so will result in a higher number of receptive females (Van Schaik 2000). In order to prevent the death of her foal under these circumstances it would be in the interest of the female to mate with her band stallion (Andersson 1994). However, like the low fidelity seen by Gray *et al.* (2012), infanticide may also be the by-product of human interference as it has rarely been reported in free-living populations that have not experienced human interference (Feist and McCullough 1976; Feh & Munkhtuya 2008). Only one attempt has been recorded; it occurred in the Virgina Mountain range in Nevada when a stallion

attacked a foal he did not sire in what appeared to be an attempt to kill it (Gray 2009). This however is the only observed incident in a population not influenced by human interference despite extensive study and observation.

Sable Island Horses

The Sable Island horses are a small group of feral horses residing on a crescent-shaped sand bar located 290 kilometers east from Halifax, Nova Scotia (Parks Canada 2012). Their population generally fluctuates between 150 and 400 individuals (Lucas et al. 1991). A study by Plante, *et al.* (2007) on their genetic diversity as compared to 15 Canadian breeds and five Spanish breeds discounted the popular theory that they arrived on the island from shipwrecked Spanish ships. Instead, it was found that they were more closely related to breeds commonly found in eastern Canada, not Spain. Their first introduction was by Andrew LeMercier, a clergyman from Boston, who stationed people on the island as well as sending livestock over. However, after his involvement with the island ended what became of the horses he brought is unknown. The horses currently on the island likely descended from a second introduction of horses put to pasture on the island by his partner, Thomas Hancock. These horses had likely originally belonged to the Acadians, who had to leave much of their belongings and livestock behind after their expulsion from Nova Scotia (Christie 1980). The horses had been managed with new horses being occasionally introduced up until the mid-1900s (Plante *et al.* 2007), when they became protected by law. The horses occasionally undergo population fluctuations, which at times may be severe. Mortalities occur most often in late winter and early spring are often associated with harsh winters (Lock 1987), although the factors influencing mortality selection during these die-off events is not known.

Based on observational study of the horses, it is assumed they remain within their home ranges and do little exploration outside of that done by bachelors despite being non-territorial, and avoid encounters with other bands. There is some overlap in home ranges of bands, as their distribution appears to be influenced by the presence of resources such as water, vegetation and shelter (Welsh 1977). Sable Island horses appear to exhibit the same mating systems as other feral horse populations, forming bands of females (harems) with an associated dominant stallion, totaling 2-10 horses, averaging 5 individuals. Most observed matings have been between band stallions and the females within his harem (Welsh 1977). Those males without harems form bachelor bands and occasionally solitary males are seen. Band stallions will drive off bachelor males that approach his harem, although if that male had previously been a part of the harem as a juvenile he may be accepted as a subordinate stallion. (Plante *et al.* 2007; Welsh 1977). Beyond band structure, the horses are grouped into three main subdivisions on the island rather than being distributed evenly across the island; separating into west, central and east locations (Contatsi *et al.* 2012), with differences in heterozygosity from one end of the island to the other (Lucas *et al.* 2009).

In 1960 the Sable Island horses were given protection under the Sable Island Regulations of the Canadian Shipping Act (Province of Nova Scotia 2014). Since then, no domestic horses have been introduced to the island and all studies have been non-invasive. These horses provide a unique opportunity to study a geographically isolated population of horses and their mating behavior free of human interference and the introduction of new individuals. Most other feral horse populations have experienced

some level of interference or management including parasite treatment, veterinary care (Kaseda and Khalil 1996), culling (Bowling and Touchberry 1990) and immunocontraception (Madosky *et al*. 2010) making Sable Island horses unique in this regard. Sampling methods in other populations have often been invasive or disruptive, involving round-ups to take blood samples to be used in molecular analyses (e.g. Kaseda and Khalil 1996; Gray 2012; Bowling and Touchberry 1990). All samples from Sable Island were taken from already deceased individuals, which avoids issues such as band disruption that have potentially occurred in other studied populations as a result of round-ups.

Study Objectives

This study aimed to assess patterns of relatedness in the Sable Island horses through the integration of field and molecular data, with the field data providing information on location and the molecular data providing data on relatedness. The impact of inbreeding within the population on mortality will be assessed through the integration of molecular data on individual heterozygosity and field data on age class of the deceased individuals used in the study. This study aims to assess the reproductive strategies of and impact of inbreeding on the horses of Sable Island, an isolated population that lacks the levels of human interference seen in other feral horse populations.

The first objective of this study was to assess social structure and mating system through the examination of patterns of relatedness within the population. Patterns of dispersal within a population can be assessed though the comparison of relatedness data to field data on location and group membership (Miyamoto *et al.* 2013) and the usage of

network analysis (Moore *et al.* 2014). Female horses are expected to exhibit some correlation between clusters of related individuals and location. Females disperse from their natal band, likely as a mechanism of inbreeding avoidance (Linklater and Cameron 2009). However, they usually still exhibit philopatry, staying in their natal areas (Linklater and Cameron 2009) and are quickly accepted into a new band instead of having to search out mates as males do (Monard *et al.* 1996). Males are expected to show little to no correlation between clusters of relatedness, as they are not expected to show the same level of philopatry as females and likely disperse farther from natal bands. Male dispersal is likely driven primarily by competition for mates (Dobson 1982), as relatively few will gain access to a harem of females. As a result increased dispersal from natal areas as compared to females is expected.

The second objective of this study was to examine the connectedness of individuals within the population to assess reproductive success and mating structure, using the same data on relatedness and the network generated from it. The differential access to mates caused the polygynous mating structure expected of Sable Island horses affects not only dispersal but reproductive success as well. Most if not all females are expected to have access to a mate while males have differential access as only few males will become band stallions (Klingel 1975). Those who fail to become part of a band during their lifetime likely have very little reproductive success, as extra-pair mating has been found to be low among horses (Kaseda and Khalil 1996) and bands are usually stable in membership (McCort 1977). Males would then show higher variance in connectedness than females due to resulting differences in reproductive success, and likely overall lower connectedness than females as only few will become band stallions.

The final objective of this study was to assess if inbreeding within the population is having a negative effect on mortality. The Sable Island horses are a small, genetically isolated population, which puts them at risk for loss of genetic diversity through inbreeding, potentially leading to inbreeding depression (Frankham 2004). If inbreeding is impacting mortality within the population, it would be expected that offspring exhibiting low levels of heterozygosity would die before reaching adulthood due to decreased fitness (Keller et al. 1994) from the expression of deleterious alleles (Ralls, Ballou and Templeton 1988), which can lead to increased parasite load (Coltman et al. 1999b) or susceptibility to environmental pressures (Keller et al. 1994). This would result in an adult population with higher levels of heterozygosity than young individuals, as young individuals will have not yet lived through many selection pressures, and juveniles are expected to have intermediate levels of heterozygosity.

Materials and Methods

Sampling

Tissue samples of skin and muscle from deceased horses on Sable Island have been collected since 1987 by a collaborator (Zoe Lucas). Those used were stored in salt-saturated 20% DMSO solution with 0.25 M EDTA (Seutin *et al.* 1991). The goal of this project was to analyze the patterns of reproductive success and genetic diversity in a representative cross-section of the population. In 2003 there was a particularly large die-off, with 255 carcasses found, and this study is based on samples from that year. This sample represents a good opportunity to look at relatedness, as it provides a good snapshot of the population at the time, encompassing individuals across multiple generations. This includes parents and offspring, so reproductive success may be evaluated from these samples. It is also a good opportunity to examine factors influencing mortality, such as inbreeding, as this is a large selection event that caused mortality across age classes in a large portion of the population.

DNA Extraction and Quantification

Forty milligrams ± 5mg of tissue were used for each extraction using skin tissue when possible. The tissue was minced finely then placed in a mortar chilled with liquid nitrogen and ground with 100μL lysis buffer (10 mM Tris, 0.1 M NaCl, 40 mM dithiothreitol (DTT), 10 mM ethylenediaminetetraacetic acid (EDTA), 2% sodium dodecyl sulfate (SDS))(Budowle et al. 2000). Samples were transferred to 1.5MmL tubes with 300μL lysis buffer. Bovine muscle placed directly in 400μL lysis buffer was used as a positive control and 400μL of lysis buffer alone was used as a negative control for each

round of extractions. Samples were left at room temperature for at least four days, upending frequently to mix. Proteinase K was added at 0.5U per milligram of tissue (33.3μL of 600U/mL) and left at room temperature overnight. The next day an additional 33.3μL of proteinase K was added and samples were placed in a 65ºC water bath for one hour then transferred to a tray of water from the water bath to cool at room temperature for one hour. One final shot of 33.3μL of proteinase K was added and samples were left at room temperature overnight.

Extraction of DNA was conducted using standard phenol:chloroform procedures (Sambrook and Russell 2001). Four-hundred microliters of phenol:chloroform were added to each sample which were then upended for five minutes and centrifuged at 12,000xg for 1 minute. The top aqueous layer was transfered to a new tube. This process was repeated again with phenol:chloroform and then with chloroform alone. The DNA was then precipitated by adding 80μL of 10mM ammonium acetate and 800μL of 95% ethanol to the sample and mixed, and then incubated at -20C overnight. The next day they were centrifuged at 12,000xg for 10 minutes and the ethanol supernatant was then carefully decanted, using a Kimwipe to draw out excess ethanol from the tube. One-hundred microliters of 70% ethanol was added, rolling tubes to ensure that ethanol touched all sides and any remaining salts were removed. Samples were centrifuged at 12,000xg for 10 minutes again and ethanol supernatant was decanted. Excess ethanol was removed using Kimwipe wrapped around pipette tip without disturbing the pellet. Samples were left to air dry for 10 minutes, after which 150μL of $TE_{0.1}$ (20mM Tris-HCL, 0.1mM EDTA (ethylenediaminetetraacetic acid), pH 8.0) was added to suspend DNA. Samples were placed in a 65ºC water bath for 5 minutes to ensure pellet was dissolved,

and then stored in the freezer (-20ºC).

The amount of DNA obtained was estimated using spectrophotometry. A NanoDrop 2000 (Thermo Scientific) was used to process standards of calf thymus at concentrations of 1ng/µL, 5ng/µL, 10ng/µL and 50ng/µL each time equipment was used to ensure accuracy of readings. Optical density readings were then obtained at wavelengths of 260 and 280nm (OD_{260} and OD_{280}) to estimate DNA quantity and purity. Aliquots of samples diluted to 5ng/µL were made based on these readings and this standardized DNA was then amplified using sexing primers Y53-3C, Y53-3D, P1-5EZ and P2-3EZ (Gilson et al. 1998). Samples were amplified in a 15µL PCR cocktail containing 1x GoTaq® Flexi Buffer (Promega), 0.4mg/mL BSA (Invitrogen), 0.2mM of each dNTP, 1.5mM MgCl2, 0.3mM of each primer, 0.05U/µL GoTaq® DNA polymerase, 1.65µL reaction water and 20ng DNA from each sample. A positive control containing 20 ng of calf thymus DNA and a negative control containing of 4µL $TE_{0.1}$ was amplified each reaction as well. PCR conditions consisted of: 94ºC for 5 minute; 30 cycles of 94ºC for 30 seconds, 55ºC for 1 minute, and 72ºC for 1 minute; and 45 minutes at 60ºC as a final extension step. After PCR, 5µL Orange G loading dye was added to each sample of which 10µL was then loaded into a 1.5% agarose gel stained with 0.05µg/mL ethidium bromide. Fifteen microliters of a MassRuler™ Low Range DNA Ladder (Thermo Scientific) was loaded on every gel to reassess quantity by comparing intensity of bands. Gels were run at approximately 100 volts for an hour and then visualized using an ultraviolet transilluminator.

Genotyping

All samples were amplified at 17 microsatellite loci using the StockMarks®
Equine Genotyping Kit (Applied Biosystems, Foster City, CA). An 11µl reaction volume
was used containing the following: 1.61µl StockMarks® PCR buffer, 2.57µl dNTP mix,
0.32µl AmpliTaq Gold® DNA polymerase, 2.57µl amplification primer mix, 1.93µl
reaction water, and 2µl of 0.5 ng/µl DNA. For each reaction, a positive control consisting
of 2 µl of a 1:80 dilution of the control DNA provided with the kit and a negative control
of 2µl of $TE_{0.1}$ were amplified. PCR cycling conditions consisted of: 95ºC for 10 min.; 30
cycles of 95ºC for 30 sec., 60ºC for 30 sec., and 72ºC for 1 min.; and a final extension
step of 72ºC for 1 hour. All PCR amplifications were conducted on Veriti® 96-well
thermal cyclers (Applied Biosystems). Amplified DNA was then prepared for capillary
electrophoresis by adding 2µl of PCR product to 10µl of HiDi™ formamide and 0.25µl
GeneScan™ 600 LIZ® size standard (Applied Biosystems). Amplified products were
then size-separated and visualized on a 3500xl Genetic Analyzer (Applied Biosystems).

Samples were genotyped using the GeneMarker® software (SoftGenetics, State
College, PA). Scoring of the size standard for all samples was examined to ensure that it
had been scored correctly and then samples were genotyped manually. Estimates of locus
characteristics and variability were obtained using CERVUS version 3.0.6 (Kalinowski
2007), and genotypes were tested for deviations from Hardy-Weinberg equilibrium using
GENEPOP version 2.2.2 (Rousset 2008). Loci were not tested for linkage, because this
kit was generated based on loci that were known to not be linked.

General Approach for Statistical Analyses

There has been increasing recognition, across a range of scientific disciplines, that there are several shortcomings in relying on p-values for hypothesis testing, and that in several situations doing so can result in incorrect conclusions about the data (Anderson *et al.*, 2001; Gigerenzer, 2004; Guthery, 2008; Kruschke, 2012). Although there is not a single method that alleviates all of the problems with p-values without introducing its own inherent limitations (Gigerenzer and Marewski, 2015), Bayesian analyses provide an attractive alternative as inferences about the data can be made in a probabilistic manner (Wade, 2000; Kruschke, 2011, 2012). Analytically complex computational resources now exist, such as R (R Core Team 2014) and JAGS (Plummer 2003), which make it possible to build analytical models and conduct Markov chain Monte Carlo (MCMC) estimation in a relatively easy manner. These tools were used for analyses throughout this thesis.

Relatedness

Using the related package for R (Pew *et al.* 2015), relatedness was estimated with the Wang relatedness estimator (Wang 2002). These estimates were used to create a weighted network which was then visualized using Cytoscape (Shannon *et al.* 2003). A cluster analysis of the network, using the "fast greedy" method (Clauset *et al.* 2004), was performed to look for groupings of related individuals. This method looks for dense subnetworks within the larger network through the optimization of modularity, which measures the strength of division within a network. Correlation between clustering and location was then analyzed; with each individual assigned to west, central or east to examine patterns of dispersal within the population. Representation of each cluster in each location was examined by splitting the dataset by area and examining frequency of

cluster membership. Transitivity, a metric measuring of degree of clustering, of both males and females was then calculated to compare.

Connectedness of males and females was measured to evaluate the number of relationships each individual had, and how related they were to them. This was done to give insight into mating structure of the horses, as those who have more offspring will have higher connectedness. Connectedness was evaluated using the metric of graph strength. This metric combines number of edges, represented by number of connections each individual has; and weight of those edges, which is determined by relatedness.

Inbreeding

Estimates of individual heterozygosity were calculated using the package GENHET version 2.3 for R (Coulon 2010) and both heterozygosity by loci and internal relatedness were used in further analyses, as well as doing the analysis for all individuals and then doing separate analyses for males and females. Internal relatedness is an estimator where the frequency of an allele within the population contributes to its weight, with rare alleles contributing more to estimates of homozygosity than common ones. It explains more variation in fitness than similar estimators, and is efficient in populations with low immigration rates (Amos *et al.* 2001). Heterozygosity by loci weighs the contribution of each loci depending on the variability of the alleles so that the most informative loci carry the most weight. It does so without discriminating against rare alleles, as internal relatedness does. It is a good alternative to estimators that use allelic frequencies alone, particularly in populations that have relatively high levels of heterozygosity. However, internal relatedness may be more efficient in some populations

showing low immigration (Aparicio *et al.* 2006), such as the Sable Island horses, so both are used here.

Based on field data, each individual was categorized as either foal (less than one year old), juvenile (one to four years old) or adult (older than four years) and those who could not be placed in a specific age group were not used. To compare the degree of difference in inbreeding between the age classes a Bayesian equivalent of an analysis of variance (ANOVA), sometimes called a BANOVA, was conducted (Kruschke, 2011). A "robust" analysis was conducted by fitting a *t* distribution to the data, which accommodates outliers better than a normal distribution. Posterior probabilities were estimated for the following parameters: the mean and standard deviation within each age class; the differences in these values between the age classes; and the effect size of each age class (the degree to which inbreeding coefficients tend to increase or decrease in relation to each age class). Prior probabilities for the model were chosen based on the recommendations in (Kruschke, 2011). They were then estimated by simultaneously running three MCMC chains with a burn-in of 10,000 steps and 20,000 saved steps, where data were recorded for every 25th step. Chain convergence and model performance were evaluated through the visual examination of trace plots and autocorrelation plots, and estimates of effective sample sizes. The model was run multiple times to ensure estimates did not change across runs.

Results

Microsatellite scoring

Of the 17 loci amplified using the StockMarks® Equine Genotyping Kit, three (HTG6, HTG7, HMS6) were not used because they could not be reliably scored. Two were not used (LEX3, ASB23) because they showed significant deviations from Hardy-Weinberg expectations. All analyses were therefore based on the remaining 12 loci (VLH20, HTG4, AHT4, HMS7, AHT5, ASB2, HTG10, HMS3, HMS2, ASB17, HMS1, CA425). Of 212 samples genotyped, 208 were used; the remaining four did not genotype well and were missing information for too many loci to be used.

Relatedness

Three relatedness clusters were found within the population when all individuals were included. When compared to the location data, little relationship between clustering and location was found (correlation coefficient = 0.0585). When examining females on their own, three clusters were found and again little relationship was found between cluster membership and location (correlation coefficient = 0.00515); however, four clusters of relatives were found in males, which showed a strong negative relationship between cluster membership and location (correlation coefficient = -0.184). The transitivity for females and males were similar, at 0.63791 and 0.6025, respectively, indicating that the degree to which individuals cluster together based on relatedness was similar between the sexes.

Connectedness

Differences in connectedness were found when comparing males and females using the metric of graph strength. Females were slightly more connected than males (Figure 1), indicating that overall they had a large number of close relatives; and males showed slightly more variation, suggesting that the number of close relatives differed more between males than between females.
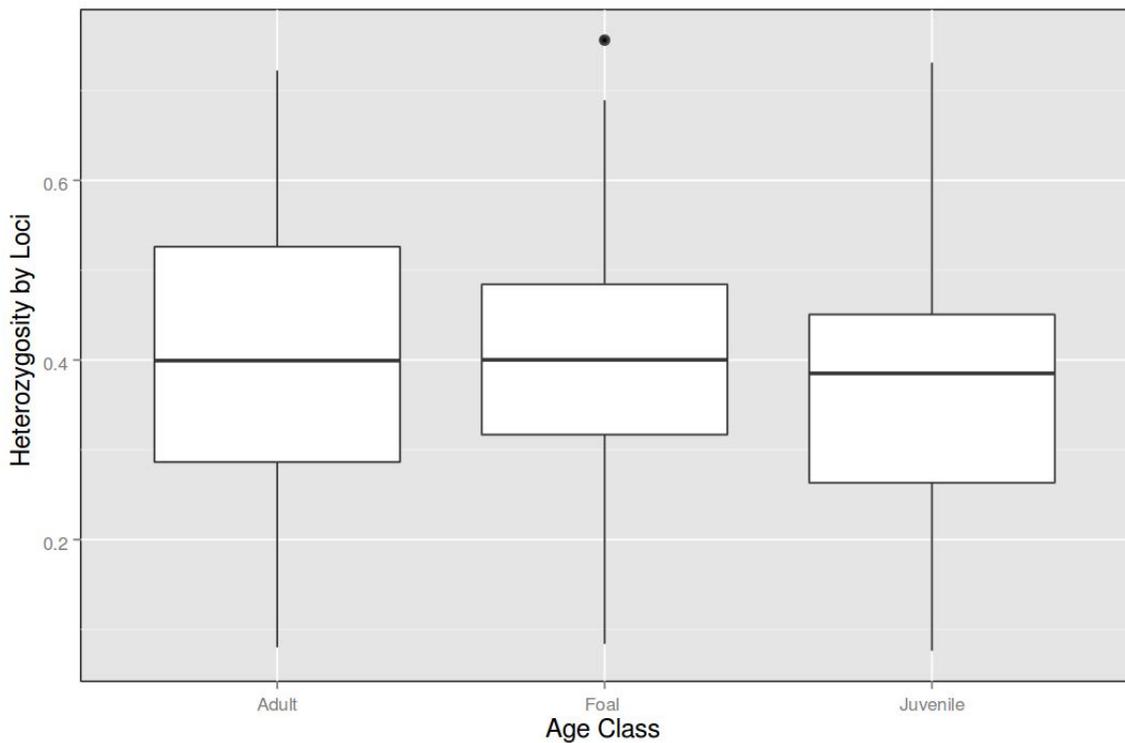

Figure 1. Connectedness of males versus females
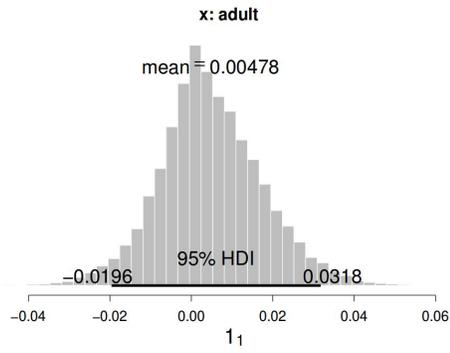
Inbreeding and Mortality

Twenty-one individuals had to be excluded from analysis as their age class could not be distinguished between juvenile and adult. Values for both estimators of individual heterozygosity showed similar results, so only values for heterozygosity by loci will be used here. There was little difference seen in inbreeding level among age classes (Figure 2), including when the data set was split into males and females for comparison. In

addition to this, the patterns expected if inbreeding was affecting mortality was not seen in that juveniles had slightly lower inbreeding levels than both foals and adults, although the difference was small. The highest density interval (HDI) of the effect size for each age class, which measured increase or decrease in inbreeding coefficient, showed low means (adult=-0.00304, juvenile=-0.00824, foal=0.0113) and overlapped zero, or no effect, in all age classes (Figure 3). This included comparisons between adults and foals (mean=-0.00826) where differences in effect size would be expected to be highest.
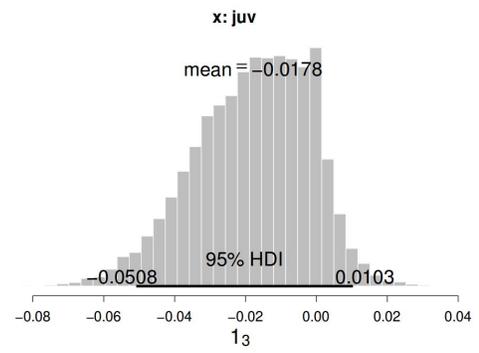

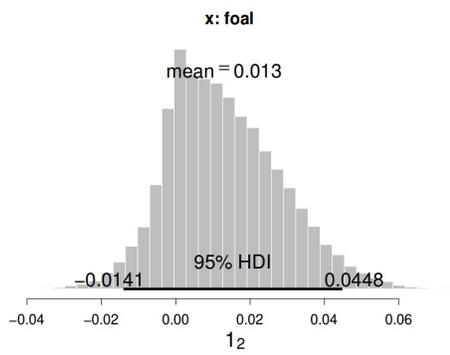**Figure 2.** Inbreeding levels across age classes in deceased individuals

a)

x: adult

mean = 0.00478

95% HDI

−0.0196   0.0318

1_1

b)

x: juv

mean = −0.0178

95% HDI

−0.0508   0.0103

1_3

c)

x: foal

mean = 0.013

95% HDI

−0.0141   0.0448

1_2

Figure 3. The highest density interval (HDI) of the effect size a) adults b) juveniles and c) foals.

Discussion

Relatedness

Clusters of relatives were seen when examining all individuals, however there was little correlation between relatedness cluster membership and location. This was also seen when examining just females where there was very low correlation between clusters of relatedness and location, indicating that groups of related females are spread throughout the island rather than staying together in one location, suggesting that they are not preferentially choosing or forming new bands in locations close to relatives. This would suggest that philopatry is low among female Sable Island horses, since if they were exhibiting philopatry a correlation between the two would be expected. This is dissimilar to what is seen among other horse populations, as previous studies have found that female horses disperse without straying far from their natal band (Linklater and Cameron 2009; Kaseda *et al.* 1997). Dispersal patterns may be influenced in part by differences in resource availability and population density between regions, as a study by Contasti *et al.* (2012) suggested that these factors affect the emigration of females between areas on Sable Island. Although the dispersal of female horses is often linked with inbreeding avoidance (Linklater and Cameron 2009), this does not appear to be the case in this population. Females would likely encounter familiar males elsewhere in the island as they are also dispersing from natal areas.

Males disperse as well, but unlike females they showed a negative correlation between relatedness cluster and location. A negative correlation implies that they are avoiding male relatives upon dispersal, rather than just dispersing throughout the island

like females. This suggests that they are actively avoiding locations containing close male relatives (Linklater and Cameron 2009) rather than just dispersing throughout the island, which would result in no correlation as seen in the females. Male dispersal is likely a result of both competition among males and inbreeding avoidance (Dobson 1982) and they may be prioritizing the avoidance of competition with male kin over other competition with unrelated males. Dispersal has also been suggested to be density-dependent (Kaseda 1991), so some males may be actively leaving natal areas with high density improve their chances of gaining access to females. Although males and females may share some potential root causes for dispersal, males experience increased levels of mate competition and difficulty forming bands as compared to females as well as increased pressure to leave from natal band members (Monard *et al.* 1996), which may encourage them to disperse farther.

Connectedness

A slight difference was seen in the connectedness between males and females (Figure 1), with males showing slightly increased variation and females showing higher overall connectedness. This is expected of the polygynous mating structure horses exhibit, as females are expected to have relatively equal access to mates as most, if not all, will have a position in a band, while males have differential access to mates (Klingel 1975). Female horses have been shown to have differential reproductive success within bands as well (Powell 2008), with dominant females sometimes restricting the access of females to the band stallion (Stebbins 1974). Most of the variation in female reproductive success is expected to result from age (Cameron *et al.* 2000) and levels of social integration (Cameron *et al.* 2009). These differences are not expected to be as large as those seen in

males, however, as almost all females are quickly accepted into bands (Monard *et al.* 1996) where they have access to mates.

The males who occupy the position as band stallion within a harem will have more offspring and thus more close relatives than those who remain solitary or in bachelor bands. Males retain their position as band stallion across multiple breeding seasons, however residency times will vary among males (Klingel 1975), contributing to greater variation in connectedness. As well infidelity is expected to be low among horses (Feist and McCullough 1976; Gray *et al.* 2012), contributing to the differences seen as bachelor males are not frequently gaining access to females through extra-pair mating, increasing the variation in reproductive success among males. Connectedness levels, which were seen to be higher in females, can also be attributed to the polygynous mating patterns of horses. Most males at any point in time will be bachelors, and therefore have lower levels of reproductive success than band stallions and most females (Klingel 1975). As a result a large proportion of males will then have little to no opportunity to breed and thus low reproductive success; this would result in the lower average connectedness seen in males.

Inbreeding

Minimal difference was seen in levels of inbreeding among the three age classes (Figure 2). The HDI of the effect size for each age class all included zero, or no effect (Figure 3), as well. These results do not support the idea that mortality is being impacted by levels of inbreeding. The levels of inbreeding among age classes do not follow the pattern expected of a population suffering from increased mortality. Foals would be expected to have the highest levels of inbreeding and adults the lowest, as adults would

likely have lived through more previous selection pressures than foals. This would remove those individuals with high levels of inbreeding from the population before reaching adulthood. The Sable Island horses go through occasional and sometimes severe population fluctuations, which are associated with harsh winters (Lucas *et al.* 1991), making it likely that most adults would have lived through at least one die-off event similar to the one seen in 2003, although likely one with fewer mortalities as the one being studied was particularly severe. The differences among age classes expected were not seen despite adults having presumably lived through previous selection pressures, particularly since juveniles exhibited the lowest levels of inbreeding of all three. A definitive conclusion about the impact of inbreeding on mortality cannot be reached, however, without examining the inbreeding levels in individuals who survived the die-off event. The expected patterns would not be seen if previous events had not been significant enough to remove individuals suffering from inbreeding depressing from the population, or if they had not been recent enough for the adults who died during the 2003 event to have lived through one. In these scenarios little difference would be seen among age classes as individuals suffering from inbreeding depression would survive to adulthood.

Even if the horses are not currently suffering from inbreeding depression, it does not exclude this from being a possibility in the future. The population size is small (Lucas *et al.* 1991) and genetically isolated as their protected status prevents new horses from being taken to the island (Province of Nova Scotia 2014). This, as well as their nonrandom mating system, puts them at risk for future decrease in heterozygosity within the population and inbreeding depression (Frankham 2004).

Future Directions

A number of future studies could be conducted to further the understanding of the Sable Island horses. With a sufficiently large sample size; used in conjunction with field data on band structure, foaling, and male residency; analysis of paternity could be undertaken. Mares, including those on Sable Island (Welsh 1975), are presumed to have high fidelity to band stallions; however no genetic studies have been done on the Sable Island horses to support this. Most molecular studies to date on paternity in feral horses involve populations that experience human interference and disruptive sampling methods (Kaseda and Khalil 1996; Gray 2012; Bowling and Touchberry 1990). This may impact behavior and band composition whereas sampling methods in the Sable Island horses are non-disruptive and they are protected by law from interference. Further investigation into potential impacts of inbreeding, such as increased parasite load (Coltman *et al.* 1999b), may also be of interest, and a comparison of inbreeding levels of individuals who died during the 2003 die-off to those who survived should also be undertaken. This study examined only mortality in comparison to age class; negative effects that are not causing increased levels of mortality within foals but still affecting the overall condition and fecundity of the horses may still be occurring as a result of inbreeding. Investigation into the cause of dispersal could be undertaken as well to examine the causes behind the differences seen in males and females, particularly the negative correlation seen in males; and whether this is a result of the increased aggression received by male foals (Linklater *et al.* 1999), the result of male mate competition (Dobson 1982), or a mechanism of inbreeding avoidance (Linklater and Cameron 2009).

Acknowledgements

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