

Population Genetics and Reproductive Indicators of Tennessee Elk

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Degree
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ABSTRACT

Two genetically distinct elk populations from Elk Island National Park (EINP) in Alberta, Canada were used for Tennessee elk reintroductions. Previous evaluation of Tennessee elk determined EINP populations persisted with possible spatial segregation and minimal admixture. Pregnancy rates were determined during initial releases; however, no updated evaluation has been conducted. We evaluated genetic variability and relatedness, assessed spatial distribution, determined pregnancy rates of captured adult females, and evaluated the efficacy of non-invasive fecal sampling for detecting pregnancy in Tennessee elk. From February through April 2019, 357 fecal samples were collected non-invasively from 65 areas across 489.62 km² [kilometers squared] of the North Cumberland Wildlife Management Area. In 2019 and 2020, blood, hair, and fecal samples were collected from 29 collared elk (21 females and 8 males). Analysis of 16 microsatellites and a sex marker from fecal samples collected non-invasively and hair from capture genetically identified 101 individuals. Individuals were assigned to EINP North ($n=21$), EINP South ($n=64$), or admixture ($n=16$) with EINP North elk exhibiting high relatedness. From GPS locations, results indicated EINP genetic groups largely exhibited spatial segregation and small annual home range sizes. Using Arbor Assays Progesterone EIA and Progesterone Metabolite kits, we determined fecal progesterone metabolite (FPM) concentrations of captured females and established a FPM concentration reference range to categorize pregnancy status of feces collected non-invasively. Pregnancy rates of captured adults using pregnancy-specific protein B testing were 72.7% and 75% in 2019 and 2020, respectively. From non-invasive fecal collection,

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INTRODUCTION

Reintroduction of wildlife species into suitable habitats has become an increasingly common practice as it relates to the success of future generations. For a species such as elk (*Cervus canadensis*), reintroduction into an area once within their historical range promotes public interest, and the establishment of new, sustainable populations. However, once a species has been reintroduced, population and individual health needs to be monitored. Elk distribution and parameters influencing population health should be continually assessed to promote population success through effective management.

Genetic variability and pregnancy rates are 2 important parameters that should be monitored to evaluate reintroduction efforts over time. Genetic variability is important for long-term success of a species as it pertains to reproduction and growth within a targeted population (Hicks et al. 2007). Small populations increase the likelihood of inbreeding. Reduced genetic variability negatively influences individual fitness and overall population growth and viability (Frankham 1995, Hicks et al. 2007). Thus, monitoring genetic diversity across multiple generations after reintroduction is important.

Pregnancy rates of adult females provides important information on population dynamics (Proffitt et al. 2014). Pregnancy-specific protein B (PSPB), a ruminant protein hormone secreted by the placenta, has shown success in determining pregnancy status using blood samples in a variety of species, including elk (Seixas et al. 2019). However, collecting blood samples requires invasive techniques and physical manipulation of an animal.

Non-invasive sampling protocols using fecal and hair samples are becoming widely used to assess reproductive parameters and provide managers with a cost-effective tool for monitoring wildlife populations when blood samples cannot be easily obtained (Miller et al. 2005, Goode et al. 2014). Metabolites of reproductive steroids, such as progesterone, are excreted in feces and concentrations of these metabolites can be measured repeatedly across gestation or across numerous individuals to determine pregnancy status (Schwarzenberger et al. 1996). Monitoring fecal hormone concentrations can be used to assess pregnancy status on both an individual and population level without the need for invasive capture (White et al. 1995).

We wanted to evaluate genetic variability and pregnancy status and rates of elk at the North Cumberland Wildlife Management Area (NCWMA) in East Tennessee using fecal samples from non-invasive landscape collection and hair, blood, and fecal samples collected from captured elk.

BACKGROUND

Elk Restoration Interest in Tennessee

Historically, the eastern elk (*C. c. canadensis*) inhabited much of the eastern portion of the United States. Due to overharvest and habitat loss, the eastern subspecies of elk was extirpated with the last reports of elk in Tennessee occurring in the late 1800's (Bryant and Maser 1982, O'Gara and Dundas 2002). Following a successful reintroduction to Kentucky in 1997 (Larkin et al. 2003), there was high public interest for reintroducing elk into Tennessee and an elk restoration plan was developed by the Tennessee Wildlife Resources Agency (TWRA; TWRA 2000). Beginning in 2000, elk

were successfully translocated to the Cumberland Mountains in Tennessee (Kindall et al. 2011).

Elk Island National Park as a Source for Elk Restoration in Tennessee

Based upon genetic similarity and extensive disease monitoring history, elk originating from Elk Island National Park (EINP), Alberta, Canada were selected as the source population for Tennessee reintroductions (TWRA 2018). Manitoban elk (*C. c. manitobensis*) which occur in EINP are the closest recognized living and genetically similar subspecies of extinct eastern elk (Polziehn et al. 1998). Large ungulate populations such as elk, plains bison (*Bison bison bison*), wood bison (*B. b. athabasca*), moose (*Alces alces*), and mule deer (*Odocoileus hemionus*) were depleted in Alberta, Canada in the late 1800s as a result of overharvest and commercially supplying meat to Europeans (Blyth 1995). In response to severe population reductions, a petition was signed in 1904 to protect remaining species in Alberta, leading to the establishment of EINP as an elk and large ungulate preserve (Blyth 1995). As a result, populations of elk, moose, plains bison, and wood bison have been continually managed at EINP for over a century (Parent 2017).

In 1907, a fence was erected around EINP and at least 24 elk were enclosed within the park (Blyth 1995). Expansion of EINP south of a major highway occurred in the 1940s and elk were enclosed in the southern section in 1947 (Blyth 1995). Management of elk at EINP eventually led to healthy, viable elk populations by the mid-1900s (Blyth 1995, Speller et al. 2014). Once established, EINP elk were used for reintroductions in the 1980s and 1990s to locations within Canada including northwest

Alberta, Manitoba, Ontario, British Colombia, Yukon, and Saskatchewan (Rosatte et al. 2007, Speller et al. 2014). EINP elk from both the north and south sections of the park were also used as stocking sources for multiple reintroductions to the southeastern United States (Murrow et al. 2009, Kindall et al. 2011).

Land Between the Lakes as a Source for Elk Restoration in Tennessee

Land Between the Lakes National Recreational Area (LBL) is located in southwestern Kentucky and northwestern Tennessee and also received elk from EINP. In 1996, a total of 29 elk were translocated to LBL and released into a fenced enclosure for public viewing. Elk brought in to LBL were a mixture of individuals translocated from both the north and south sections of EINP in 1996, 1997, and 2002 (reviewed in Muller et al. 2018). Elk from LBL, originally sourced from EINP, were also used in the reintroductions at the NCWMA (Kindall et al. 2011).

Tennessee Elk Reintroduction

In 2000, 271,139 hectare Elk Restoration Zone was established in the Cumberland Mountains of Tennessee by the TWRA and centered around the NCWMA (TWRA 2000). The NCWMA is a mixed-mesophytic forest with 79,318 hectares of TWRA owned public land and leased private land and approximately 86% deciduous forest, 12% wildlife openings (pastures, grasslands, and reclaimed coal strip mines), and 1% cropland (TWRA 2018). Elevation and slope percentages range from 312.4–1,016.9 m and 0–229.56%, respectively (USGS 2017). Landscape cover across the NCWMA provides a mix of dense forest, managed wildlife openings, and planted food plots. Managers use

forest management, specifically prescribed fire, to create and improve elk foraging areas (TWRA 2018).

Between 2000 and 2008, 201 elk were captured from LBL and both the north and south sections of EINP for translocation to NCWMA. Between 2000 and 2003, 167 elk mixed from both sides of EINP were initially translocated to the NCWMA. In 2008, 34 elk were brought in from LBL (Kindall et al. 2011). Previous genetic evaluation indicated 2 genetically distinct populations from the north and south sections of EINP with minimal admixture (Muller et al. 2018).

One of the primary goals of restoring elk to Tennessee was to provide opportunities for recreational viewing and sustainable hunting (TWRA 2018). The first elk hunting season began in 2009 and as of 2021, 15 bull tags are allocated to hunters through a quota permit or conservation raffle. Since reintroduction and with ample recreation opportunities, elk have and continue to bring tremendous economic value to the state (Chapagain and Poudyal 2020).

Genetic Variability

The use of genetic data has become a fundamental tool for understanding and evaluating wildlife populations for management and conservation (DeYoung and Honeycutt 2005). Population genetics involves the frequency and interaction of genes and alleles which can be altered by evolutionary processes such as gene flow, mutation, and natural selection (Kim and Sappington 2013). All organisms have genomic information which is stored in DNA, transcribed into RNA, and translated into proteins, the biochemical basis of heredity (Bromham 2016). Genetic variation is characterized by the

percentage of heterozygous loci within an individual, and can be quantified to evaluate genetic parameters influencing a population (Lacy 1997).

Mutations developing within a DNA sequence are the foundation in which evolution and speciation can occur across generations. Mutations can be positive, detrimental, or unnoticeable. Mutations are heritable, permanent changes to the genome that are passed to an offspring when the genome is copied (Bromham 2016). Through DNA mutations, we can compare sequences, identify individuals or family groups, and study trait heritability across generations (Bromham 2016). Changes occurring at the genomic level can create new heritable material. During the process of DNA duplication, fine scale rearrangements may occur causing distinct patterns of genes to emerge (Bromham 2016).

In the process of reforming a double stranded DNA molecule, short repeated sequences are created when a copy of a DNA sequence is inserted across various locations within the genome. When a new copy is inserted next to an original sequence, tandem repeats develop due to unequal DNA exchange, or crossing over, during recombination (Bromham 2016). Tandem repeats make up satellite DNA and are thought to be generated primarily by slippage during replication (Bromham 2016). Short tandem repeats, also known as microsatellites, are small repeated sequences of 1–6 nucleotides with a repeating region typically varying between 5–40 repeats and are easily detected in the genomes of organisms (Li et al. 2002, Selkoe and Toonen 2006, Bromham 2016).

Microsatellites are frequently used for individual identification and population level assessment. Due to the high mutation rate of tandem repeats, counting the number

of repeats at various loci can provide a tool for distinguishing and comparing unique individuals (Bromham 2016). Detecting population changes can be evaluated through both large satellite loci (minisatellites), or small satellite loci (microsatellites). However, minisatellites have a higher number of repeats, typically require numerous samples for accurately assessing allelic frequencies, and have higher mutational instability (Bromham 2016). Microsatellites provide a more simplistic approach for individual or population identification due to shorter size of repeat regions and are abundant in eukaryotic genomes (Brohamn 2016). Additionally, species-specific primers have been developed for microsatellite analysis which can be used in other similar species for genetic identification (Bromham 2016). For example, highly polymorphic microsatellites originally detected in sheep and cattle were also detectable in elk using polymerase chain reaction testing (Talbot et al. 1996, Muller et al. 2018). Therefore, microsatellites are a highly variable, commonly used genetic marker for evaluating genetic variability.

Analyses using microsatellite markers have been conducted on many species for evaluating genetic relatedness, heterozygosity, and diversity including bighorn sheep (*Ovis canadensis*; Barbosa et al. 2021), caribou (*Rangifer tarandus*; Yannic et al. 2014), and white-tailed deer (*Odocoileus virginianus*; Nelson et al. 2021). In various elk populations across the western and eastern United States, genetic diversity has been evaluated in Rocky Mountain elk (*C. c. nelsoni*; Hicks et al. 2007), Tule elk (*C. c. nannodes*), Roosevelt elk (*C. c. roosevelti*), and Rocky Mountain elk in California (Meredith et al. 2007), and reintroduced populations in Kentucky (Youngmann et al. 2020) and Tennessee (Muller et al. 2018). Genetic diversity across elk populations is

variable and highly dependent on the gene flow, population size, reintroduction efforts, and overall connectivity among populations or landscape features (Meredith et al. 2007, Youngmann et al. 2020, Batter et al. 2021).

Hair and fecal samples contain a rich source of DNA that can be evaluated for genetic monitoring at an individual or population level (Creel et al. 2003, Maudet et al. 2004, Brinkman et al. 2011, Goode et al. 2014, Murphy et al. 2015). Cells from hair roots or sloughed intestinal epithelia cells lining the outer portion of fecal pellets can be easily used for microsatellite analysis of herbivore species (Waits and Paetkau 2005). These samples can be obtained through capture or non-invasive collection for large-scale, population level genetic evaluation.

Research integrating genetic methods can provide valuable information on demographic parameters (DeYoung and Honeycutt 2005). With the reintroduction of elk to the NCWMA, continual monitoring on a genetic level allows managers to assess genetic trends since initial reintroduction. Assessing genetic variability and relatedness in conjunction with GPS data collected from collared individuals and locations of non-invasively collected fecal samples can provide an updated genetic evaluation and more insight into spatial distribution of individuals.

Pregnancy Rates and Fecal Hormone Analysis

Reproduction is crucial for the success of a population and continuation of a species. Reproduction is an energetically demanding trait that competes with survival. Therefore, the energy required for important life history traits such as survival and reproduction must be strategically optimized (Morano et al. 2013). Elk are a long-lived

species with multiple opportunities to reproduce; therefore, emphasis on adult survival causes more variable reproductive output (Morano et al. 2013). Reproduction is influenced by many factors which can impact individual and overall population success. Density dependence or environmental conditions highly influence reproductive performance (Proffitt et al. 2014). Risk-sensitive reproductive allocation can maximize individual success for survival and reproduction, which are necessary for population sustainability (Gaillard et al. 1998, Gaillard et al. 2000, Garrott et al. 2003, Morano et al. 2013, Proffitt et al. 2014).

Evaluation of pregnancy is important for understanding population health and growth. Elk are considered capital, seasonal breeders with rut beginning in mid to late September and only lasting a few weeks (Hudson and Haigh 2002, Cook et al. 2004, Nussey et al. 2009, Morano et al. 2013). Once bred, a female will undergo a gestational period around 246 days and calve during the summer around June (Morrison et al. 1959). Pregnancy can be measured through hormone concentrations using various biological matrices including blood, feces, and urine (Cain et al. 2012). Hormones, such as progesterone, are secreted in high concentrations and steadily increase as the gestational period progresses. Therefore, evaluating progesterone concentrations across multiple females and the gestational period can provide a useful method for determining pregnancy status and rates of elk on a population scale.

Progesterone

Progesterone is a C 21 progestin steroid hormone found in a variety of tissues with many physiological functions. Mainly circulated in bloodstream, progesterone plays

a major role particularly in the reproductive functions of females during ovulation, implantation, and pregnancy (Graham and Clarke 1997). In mammals, the ovary is the major site of synthesis and secretion of progesterone causing cyclical fluctuations until a corpus luteum is formed and becomes the primary producer of progesterone during early pregnancy (Graham and Clarke 1997). In the early stages of gestation, the corpus luteum will regress and hormone biosynthesis for the maintenance of pregnancy is facilitated primarily by the placenta with some secretion still occurring from the corpus luteum (Graham and Clarke 1997). Prior to parturition, progesterone also functions in the mammary glands for lobular-alveolar development for milk secretion and suppression of milk protein synthesis (Graham and Clarke 1997). Other functions of progesterone indirectly related to pregnancy include facilitating sexually responsive behavioral signals in the brain and serving as a precursor for androgens and glucocorticoids (Graham and Clarke 1997, Taraborrelli 2015). Progesterone concentrations remain high throughout gestation and drop significantly after parturition (Litwack 2018). Thus, pregnancy status can be evaluated by quantifying progesterone concentrations in biological samples during the gestational period.

Pregnancy-specific protein B (PSPB) is a ruminant protein hormone which is produced in the binucleate cells of the placenta (Duquette et al. 2012). The use of PSPB testing is one of the most accurate measures for determining pregnancy status in various wild and domestic ruminant species including elk (Noyes et al. 1997, Seixas et al. 2019) moose (*Alces alces*; Huang et al. 2000), white-tailed deer (*Odocoileus virginianus*; Duquette et al. 2012), and dairy cattle (*Bos taurus*; Romano and Larson 2010). Reports

indicate PSPB tests have a sensitivity of 99% and specificity of 95% at 40 days post conception (Noyes et al. 1997, Seixas et al. 2019). Since PSPB is so highly successful in determining pregnancy, it is widely recommended and considered the standard for pregnancy determination. However, PSPB can only be measured from a blood sample collected through invasive capture and represents a hormone concentration at a single point in time.

Blood samples are the optimal method for measuring true concentrations of circulating steroid hormones. However, obtaining samples require invasive capture which negatively affects hormone concentrations (Touma and Palme 2005, Peter et al. 2018). Collecting blood samples to determine pregnancy has been difficult in wild ungulates (Messier et al. 1990, Cain et al. 2012). Physical restraints can increase mortality, disrupt reproductive success, and alter the endocrine profile of an individual (Messier et al. 1990). In white-tailed deer, reports indicated high concentrations of progesterone excreted by the adrenal gland during high stress events such as capture (Plotka et al. 1982). Thus, progesterone measured from blood samples collected using invasive methods may not be an accurate representation of concentrations reflecting pregnancy status, especially in an ungulate prey species.

Fecal Progesterone Metabolites

The development of non-invasive techniques have helped circumvent invasive capture and the need for a blood sample (Peter et al. 2018). Measuring fecal progesterone metabolites (FPM) using non-invasive fecal sampling provides an alternative method to measure hormone concentrations for pregnancy determination (Peter et al. 2018). Fecal

progesterone metabolites are released into circulation and inactivated by the liver through conjugation (Senger 2012, Miller 2017). Two major changes occur when molecules are inactivated: the saturation of double bonds and the attachment of a sulfate or glucuronide residue (Senger 2012). During the process of inactivation, or metabolization, the hormone is secreted, passed through and inactivated by the liver, recirculated in the blood, enters the kidney, and is excreted in urine or back into the liver and passed with bile into feces as glucuronide or sulfate metabolites (Schwarzenberger et al. 1997). Many metabolites in the glucuronide form are excreted into urine as the inactivated hormone molecules are water soluble and can easily be carried into the bladder. For metabolites excreted in feces, conjugated hormones enter the bile duct through the gut and may be subjected to enterohepatic circulation for recycling or passed through the intestinal passage to the rectum (Schwarzenberger et al. 1997). Due to the time required for intestinal passage, there is an associated lag time with FPMs. For ruminant species, FPM lag time has been reported to be approximately 12–24 hours (Schwarzenberger et al. 1997). Despite this lag time, concentrations of FPM show similar hormone profiles as those within plasma and milk, reflect placental function, and can be easily obtained and analyzed without physical manipulation of an individual (Schwarzenberger et al. 1997).

Many laboratory techniques have been developed for assessing pregnancy hormone concentrations using radioimmunoassay (RIA) and enzyme immunoassay (EIA; Haddad et al. 2019). Radioimmunoassay techniques have been used commonly in the past; however, they require the use of radioactive hormones, are costly, and require specialized laboratories and equipment necessary for testing. Competitive EIAs have

become more commonly used for hormone assessment due to the cost effectiveness and ease of use across a wide variety of species and sample types (Haddad et al. 2019). There are numerous strategies and applications for EIA tests; however, competitive EIAs are most preferred when detecting metabolites of steroid hormones in feces. Competitive EIAs have high sensitivity of small concentrations of antigens within complex biological samples and tests are cost effective and provide clear, straightforward results with the simplistic use of color changing enzymes linked to a specific antibody (Senger 2012, Miller 2017).

While PSPB cannot be measured in feces, it can be used as a biological validation with a blood sample when evaluating FPM concentrations. Therefore, FPM concentrations in feces can be compared to PSPB in blood when samples are collected at the same time, from the same individual. Fecal progesterone metabolite concentrations from known non-pregnant and pregnant captured females can provide a FPM concentration reference range to categorize FPM concentrations of females with an unknown pregnancy status. We can determine female fecal samples collected non-invasively using genetic techniques. Thus, using non-invasive fecal sampling can provide a stress-free method for population level pregnancy assessment and provide foundational knowledge for a fundamental reproductive parameter.

OBJECTIVES

We wanted to evaluate genetic variability and relatedness, spatial distribution of the genetic groups, pregnancy rates, and the efficacy of non-invasive fecal sampling for determining pregnancy status of elk at the NCWMA. The goals of this research were to

provide updated and additional information on important parameters of NCWMA elk population demography: genetics and reproduction. The specific objectives were to: (1) evaluate genetic variability and relatedness and compare to initial known genetic profiles of elk in Tennessee, (2) assess the possibility of spatial segregation contributing to persistence of the genetic groups, (3) determine pregnancy rates of collared adult females, and (4) evaluate the efficacy of non-invasive fecal sample as a method for determining pregnancy status of females using FPM concentrations.

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CHAPTER I
SPATIAL SEGREGATION AS A POTENTIAL INFLUENCER ON
GENETIC STRUCTURE OF TENNESSEE ELK

A version of this chapter will be submitted to the Journal of Wildlife Management for publication by Eryn M. Watson, Katherine A. Kurth, Dailee L. Metts, Brad F. Miller, Brittany L. Slabach, John T. Hast, John J. Cox, Richard W. Gerhold, Sarah E. Moorey, and Lisa I. Muller.

Eryn M. Watson collected and analyzed data and wrote the article. Katherine A. Kurth and Dailee L. Metts assisted with data collection. Brittany L. Slabach, John T. Hast, John J. Cox provided genetic data. Sarah E. Moorey, Brad F. Miller, and Lisa I. Muller assisted with manuscript preparation.

ABSTRACT

Previous genetic evaluation of the Tennessee elk population at the North Cumberland Wildlife Management Area (NCWMA) found 2 distinct lineages related to the original source populations from north and south sections of Elk Island National Park (EINP) in Alberta, Canada. Persistent genetic structuring and possible spatial segregation occurring between the EINP genetic groups. We evaluated genetic variability using DNA analysis of 16 microsatellites from fecal samples ($n=357$) collected on the landscape in 2019 and hair collected from satellite-GPS-collared elk ($n=29$) in 2019 and 2020 with programs STRUCTURE and CERVUS, and to continue monitoring of NCWMA elk We evaluated spatial distribution, home range size, and percentage of home range overlap using GPS locations from collared elk using the Spatial Analysis package in ArcGIS. We compared simultaneous distances and pairwise relatedness of elk identified during landscape fecal

collection using the R package related. We identified 101 individuals genetically assigned to north EINP (EINPN; $n=21$), south EINP (EINPS; $n=64$), or admixture ($n=16$) using a 2-population assumption and 3 populations with the inclusion of data from nearby herds from Kentucky elk ($n=295$). The Kentucky elk were originally sourced from the western United States. Relatedness of the NCWMA population was overall low (0.10); however, within group relatedness was higher for EINPN (0.23), than for EINPS (0.03) or admixture groups (0.03). We found female pairs assigned to EINPN-EINPN, EINPS-EINPS, and EINPS-admixture genetic groups in closer proximity were more closely related ($P \leq 0.001$) and high proportions of annual and breeding season home range overlap (41.02–66.08%) in collared female-female groups. Overall mean annual home range size for EINPN males was $1.43 \pm 0.24 \text{ km}^2$ and females $2.67 \pm 0.24 \text{ km}^2$. Overall annual home range size for EINPS males was $1.79 \pm 1.51 \text{ km}^2$ and females $3.71 \pm 0.78 \text{ km}^2$. Our results indicate small home range sizes, increased genetic relatedness in EINPN elk, and predominant spatial segregation of the EINP groups which may have implications on continued genetic structure of the NCWMA population.

INTRODUCTION

Genetic health has become an increasingly important parameter for monitoring wildlife reintroduction efforts and population success. High levels of genetic variability increase population fitness to better allow for sustainability and adaptation within a changing environment (Lacy 1997). Genetic variation is typically represented by the percentage of heterozygous loci in an individual (Lacy 1997). Issues from a loss of heterozygosity, typically due to inbreeding, can have negative consequences for

reintroduced species or those with small founder populations (Lacy 1997, Hicks et al. 2007). Small founder population sizes can result in increased inbreeding and low genetic variability, leading to lower fecundity and survival which negatively impact growth rates, population success, and persistence (Kardos et al. 2021).

Hair and fecal samples contain high quantities of DNA and are the 2 predominant sample types used for genetic identification (Waits and Paetkau 2005). Cells from hair roots have high DNA yields, amplification success rates, and require minimal effort for effective storage and sample preservation (Waits and Paetkau 2005). Fecal DNA can be extracted from sloughed intestinal epithelia cells lining the outer portion of fecal pellets of herbivore species (Waits and Paetkau 2005). Compared to hair, fecal DNA has lower success rates due to fecal extract inhibitors on DNA amplification and storage and preservation requirements necessary for maintaining DNA quality (Waits and Paetkau 2005). Additionally, factors including environmental conditions, sample age, and season can impact fecal DNA quality and success rate (Piggott 2004). However, feces are more easily obtained in the environment, can be collected non-invasively, and have been successfully utilized in a variety of ungulate studies (Yamauchi et al. 2000, Brinkman 2009, Brinkman et al. 2011, Goode et al. 2014). Therefore, using hair and fecal samples to evaluate genetic markers can provide valuable information on spatial and temporal changes in genetic structure within a population.

Microsatellites are tandem repeats of 1–6 nucleotides found at high frequencies in the nuclear genomes of most taxa (Selkoe and Toonen 2006). Loci vary between 5 and 40 repeats in length and are flanked by identifying sequences generally conserved across

individuals of the same species (Selkoe and Toonen 2006). Microsatellites can be used for individual identification, determining genetic group assignment, and evaluating genetic parameters with direct impacts on population health. For evaluating long term reintroduction efforts and assessing population genetic structure and demographics, microsatellites have been used across numerous ungulate species including white-tailed deer (*Odocoileus virginianus*; Goode et al. 2014), red deer (*Cervus elaphus*; Nussey et al. 2005), mountain goats (*Oreamnos americanus*; Poole et al. 2011), bighorn sheep (*Ovis canadensis*; Love Stowell et al. 2020), and moose (*Alces alces*; DeCesare et al. 2020).

Beginning in the early 2000s, 201 Manitoban elk (*C. c. manitobensis*) were reintroduced to the North Cumberland Wildlife Management Area (NCWMA) in East Tennessee (Kindall et al. 2011). The source population for the translocation was from Elk Island National Park (EINP) in Alberta, Canada. The Manitoban subspecies of elk was selected for reintroduction as it is the closest recognized genetic subspecies to the extinct eastern elk (*C. c. canadensis*; Bryant and Maser 1982); EINP has been shown to contain the Manitoban subspecies (Polziehn et al. 1998). Elk were from 2 isolated populations high fenced within EINP including the north (EINPN) and south (EINPS) sections. Elk were fenced and isolated within the north section of the park in 1907 and the south section in 1947 (Griffiths 1979, Blyth 1995). Due to the fencing, the 2 EINP populations have been geographically and physically isolated with no natural movement between either section.

Elk sourced for reintroduction were captured from both EINP populations, mixed at a central handling facility, marked, and translocated to Land Between the Lakes (LBL),

Kentucky and the NCWMA (Muller et al. 2018). Between 2000 and 2003, 167 elk were sourced directly from EINP populations and in 2008, 34 elk (originally translocated from EINP) were sourced from LBL (Kindall et al. 2011). Genetic analysis of the translocated elk identified 2 distinct groups based on whether elk were originally captured from the north or south side of EINP (Muller et al. 2018).

Analysis 11+ years after initial release of elk in NCWMA identified the persistence of the 2 genetically distinct EINP populations and assessed spatial structure of EINP groups using very high frequency (VHF) telemetry methods with coarse locations (Muller et al. 2018). As elk acclimated to the new landscape there appeared to be some spatial segregation and movement differences between the 2 groups. However, no definitive reason was found for the continued persistence of genetic structuring. Therefore, the objectives of this study were to continue monitoring the sympatric genetic groups for genetic variability, relatedness, spatial distribution, home range size, and overlap to evaluate possible mechanisms facilitating ongoing population structure.

STUDY AREA

We conducted research at the NCWMA located in East Tennessee. In 2000, the Tennessee Wildlife Resources Agency (TWRA) established a 271,139-hectare Elk Restoration Zone with the NCWMA being the primary location for elk reintroductions (TWRA 2000). The NCWMA was a mixed-mesophytic forest with 79,318 hectares of public and leased private land managed by TWRA. Primary vegetation types were 86% deciduous forest, 12% wildlife openings (fields, grasslands, and reclaimed coal strip mines), and 1% cropland (TWRA 2018).

We used samples collected from captured Kentucky elk (KY) during part of our genetic analysis for comparison (Slabach 2018, Hast 2019). A 16,802 km² elk restoration zone was established to facilitate elk reintroductions from 1997–2002 within the Cumberland Plateau physiographic region of southeastern Kentucky (Maehr et al. 1999, Kentucky Department of Fish and Wildlife Resources 2016). The elk restoration zone was a mixed-mesophytic forest within 16 counties bordering the Tennessee elk zone, Virginia, and West Virginia (Hast 2019). The area consisted of deep drainages, narrow valleys, and winding ridges and approximately 80% deciduous forest, 10% active and reclaimed surface mine, 9% cleared or agricultural areas, and 1% urban matrix (Cox 2003, Kentucky Department of Fish and Wildlife Resources 2016). From 1997–2000, Kentucky received a total of 718 Rocky Mountain elk captured and translocated from Arizona, Utah, Kansas, North Dakota, and Oregon (Larkin et al. 2003).

METHODS

During 2019 and 2020, we captured Tennessee elk using corral traps and free darting with chemical immobilization within wildlife openings in the western Elk Hunt Zones and Sundquist unit of the NCWMA. We anesthetized elk with an intramuscular (IM) injection of 2 mL BAM (1mL is comprised of 27.3 mg of butorphanol, 9.1 mg of azaperone, and 10.9 mg of medetomidine; ZooPharm, Windsor, Colorado) for females and small males and 3 mL for large males administered by darts (PneuDart Inc., Williamsport, Pennsylvania) with a Dan-Inject CO2 Rifle (Dan-Inject, Kolding, Denmark). Elk were continuously monitored under anesthesia and vitals were recorded every 5–10 minutes. We administered supplemental oxygen intranasally if oxygen

saturation levels fell below 90%. We fitted individuals with a Lotek Lifecycle Pro 500 GPS or LiteTrack Iridium 500 TL collar (Lotek Wireless Inc., Ontario, Canada) and programmed collars to record locations every 5 hours (Lifecycle Pro 500) or 150 minutes (LiteTrack Iridium 500 TL). Data from all collared elk were downloaded from the Lotek website from time of deployment until December 2021. We discarded 2-dimensional fix statuses and dilution of precision values >4 to improve location accuracy (Uno et al. 2010, Jung et al. 2018). We collected hair with the root from individual elk and stored samples in a labeled coin envelope for genetic analysis. Elk were reversed with an IM injection of 4 mL Atipamezole (25 mg/mL; ZooPharm, Windsor, Colorado) and 0.5 mL of Naltrexone (50 mg/mL; ZooPharm, Windsor, Colorado) for females and small males and 6 mL Atipamezole and 0.5 mL of Naltrexone for large males and monitored elk until fully recovered.

From February through April 2019, we collected fecal samples using a clustered sampling design in areas ($n=65$) within wildlife openings. We searched each area following a continuous 3,378 m long and 2 m wide transect in weekly intervals. Areas smaller than the transect distance were searched in their entirety. We established a 1-week resting period between sampling an area to allow time for fresh feces to reaccumulate. To reduce the chance of collecting samples with degraded DNA, we postponed sampling 1-day after heavy precipitation events (Brinkman et al. 2009). Based on fecal sampling protocols in similar species, we rated feces freshness on a scale of 1–5 (1 being the oldest and 5 being the freshest) and only collected samples rating a 4 or 5 (Kirchhoff et al. 1998, Lupardus et al. 2011, Goode et al. 2014). Upon identifying a fresh

fecal sample, we recorded a GPS coordinate using a Garmin eTrex GPS (Garmin, Olathe, Kansas), collected 10–15 pellets from each fecal pile, and stored samples in a freezer (-20° C) until analysis. To obtain elk DNA from feces, we removed samples from the freezer to thaw for 15 minutes at room temperature, lightly rubbed the outer portions of 4 pellets with a flat toothpick, and stored toothpicks in a labeled coin envelope. Hair and 2mm ear punch tissue samples were collected from captured KY elk in 2013 and 2014 (Slabach 2018, Hast 2019). Details of KY capture and collection methods are described in Slabach (2018) and Hast (2019).

Wildlife Genetics International (WGI; Nelson, British Columbia, Canada) analyzed DNA from hair and tissue samples from KY elk (Slabach 2018, Hast 2019), hair from captured NCWMA elk, and 3 toothpicks per NCWMA fecal sample using 16 microsatellites (*BL42*, *BM203*, *BM3507*, *BM4028*, *BM4107*, *BM4513*, *BM6506*, *BM888*, *BMC1009*, *CSSM041*, *Oar FCB193*, *INRA107*, *OvirH*, *Rt1*, *Rt7*) and *amelogenin* to determine sex. Personnel from WGI extracted samples following manufacturer instructions using Qiagen's DNeasy Tissue kits (Qiagen, Valencia, CA, USA) and purification methods as previously described in Woods et al. (1999) and Paetkau (2003). Standard cycling and buffer conditions for polymerase chain reaction (PCR) were performed and PCR products were visualized using an Applied Biosystems 310 or 3130 xl DNA sequencer (Applied Biosystems, Grand Island, NY, USA) and through capillary electrophoresis (Paetkau et al. 1998). Wildlife Genetics International followed protocols outlined in Paetkau (2003) to assess genotyping error-checks and prevent false identification of individuals.

We used a Bayesian clustering algorithm in program STRUCTURE 2.3.4 (Pritchard et al. 2000) to determine genetic assignment of individuals and evaluate current population structure. We determined burn-in and run length based upon previously reported methods, convergence, and minimal variability in group membership probability (q) values among runs (Muller et al. 2018). We used a 3-population assumption based on Muller et al. (2018; EINPN, EINPS, and KY) with a burn-in of 1,000,000 and a run length of 1,000,000 Markov chain Monte Carlo steps. A $q \geq 0.8$ designated samples to 1 particular genetic population (Crompton et al. 2008, Muller et al. 2018). Elk that did not have a $q \geq 0.8$ for 1 specific group were designated as admixture of all assumed populations. Sample locations were not considered during the STRUCTURE analysis. We only included KY data during the STRUCTURE analysis. To assess spatial distribution of the genetic groups using all collared elk, we used a minimum of 50 GPS fixes obtained from the time of collar deployment in 2019 or 2020 to December 31st 2021.

Further genetic analysis and spatial locations were based only on NCWMA data. We used program CERVUS 3.0 (Kalinowski et al. 2007) to evaluate observed and expected heterozygosity, number of alleles per locus, and overall mean number of alleles/locus. Deviance from Hardy-Weinberg equilibrium for each locus was assessed using a chi-square test with Bonferroni correction and compared to previous reports (Nei 1987, Weir 1996). We calculated F-statistics to estimate partitioning of genetic variance between groups (pairwise F_{ST}), the proportion of total genetic variance of subpopulation relative to total genetic variance (F_{ST}), and inbreeding coefficient (F_{IS} ; Weir and

Cockerham 1984, Williams et al. 2004, Meredith et al. 2007) in R using the package Hierfstat (Goudet 2005, R Core Team 2021). For hypothesis testing, we used bootstrapping across loci to base significance on estimated 95% confidence intervals (de Meeûs and Goudet 2007). We used the R package related (Pew et al. 2015) to estimate genetic relatedness of elk identified from hair and feces using allelic frequencies to calculate pairwise and within group relatedness (r) based upon group assignment (EINPN, EINPS, and admixture). We selected the estimator from Lynch and Ritland (1999) to calculate pairwise relatedness (r_{xy}). We chose this estimator based on performance under conditions using highly polymorphic loci and population substructure and the ability to account for inbreeding (Gear et al. 2010).

We used the Proximity tool in ArcMap 10.8 (ESRI 2019) to calculate simultaneous distances between elk pairs identified from weekly landscape fecal collection. We used a linear regression model in the package nlme (Pinheiro et al. 2013) in R (R Core Team 2021) to assess whether an association existed between distance (dependent variable) and pairwise relatedness, sampling week, sex, and genetic group as fixed effects. Individual elk were assigned as a random effect. We obtained p-values for the fixed-effects using Analysis of Variance (ANOVA) and designated statistical significance at $P < 0.05$.

We calculated 95% annual home range size and percentage of home range overlap for collared elk using the kernel density tool in the Spatial Analysis package in ArcMap 10.9 (Kolbe and Weckerly 2015, ESRI 2019). Elk had at least 100 GPS fixes from the time of collar deployment until the last day of the calendar year (December 31st) or

annually from January 1st to December 31st. We also calculated a 95% seasonal home range size and percentage of overlap for elk with at least 10 GPS fixes during September and October, coinciding with the breeding season. We used the default bandwidth and calculated kernel function based on Silverman (1986). All elk were merged into a single feature layer and home range overlap was determined to the nearest km² (Kolbe and Weckerly 2015). We determined percentage of home range overlap of elk pairs by calculating overlap area of 2 home ranges, dividing by mean home range size of each individual, and multiplying by 100 (Webb et al. 2011). We used a linear mixed model in the package lme4 (Bates et al. 2015) in R (R Core Team 2021) to assess whether an association existed between home range overlap (dependent variable) and pairwise relatedness, sex, year, and genetic group as fixed effects. Individual elk were assigned as a random effect. We used ANOVA and designated statistical significance at $P < 0.05$. We evaluated whether genetic assignment of the current population differed from genetic assignment of elk originally released at the NCWMA using a Pearson's chi-squared test with Bonferroni correction to identify where differences in genetic assignment occurred (Pearson 1900).

RESULTS

At the NCWMA, we collected a total of 29 hair samples from captured elk (21 females and 8 males) and 357 fecal samples collected on the landscape across 9 weeks. For the STRUCTURE analysis, we included 295 unique individuals from the KY elk population (Slabach 2018, Hast 2019). Due to poor performance halfway through initial genotyping of NCWMA samples, WGI removed 1 locus (*Rt13*) from the microsatellite

analysis. Genotyping success rates were 100% (21/21) for NCWMA hair, 44% (157/357) for NCWMA fecal, and 97% for KY ear tissue samples (Hast 2019). We identified 101 unique individuals (75 females and 26 males) from hair and fecal samples at NCWMA. We used 15 microsatellites for STRUCTURE and CERVUS analyses of NCWMA elk. CERVUS results indicated locus *INRA107* significantly deviated from Hardy-Weinberg equilibrium and locus *Rt7* had a minimum frequency of <3 (Table 1.1); however, we retained locus *INRA107* and *Rt7* for a direct comparison between the current study and the previous evaluation. For NCWMA elk, expected heterozygosity (H_e) was 0.50 with 3.9 alleles/locus (Table 1.1).

STRUCTURE results indicated 3 distinct genetic clusters: EINPN, EINPS, KY with admixture (Figs 1.1 and 1.2). At $K = 3$, 21.8% (22/101) individuals were assigned to EINPN, 61.4% (62/101) to EINPS, and 16.8% to admixture (17/101). Two elk identified from fecal collected and assigned to admixture exhibited strong KY influence with genetic assignment of 79.2% KY (9.1% EINPS and 11.7% EINPN) and 77.5% KY (9.4% EINPS and 13.1% EINPN) and were located in the Sundquist unit. Kentucky influence was also seen in 2 other elk: (18.6% KY 35.2% EINPS, 46.3% EINPN; 14.9% KY, 6.4% EINPS, 78.7% EINPN) located in the Sundquist unit and southwestern Elk Hunt Zones. Genetic assignment of original NCWMA elk released in TN was 52.1% (87/167) EINPN, 40.1% (67/167) EINPS, and 7.8% (13/167) admixture (Muller et al. 2018). We identified a difference in genetic assignment between the current study and during the original 3 releases with assignment decreasing for EINPN ($P < 0.0001$) and increasing for EINPS

($P=0.0046$). No significant difference was identified for admixture ($P=0.085$); however, we did assign a greater proportion of elk to admixture as compared to original releases.

Overall we found low genetic differentiation ($F_{ST} = 0.01$) and low degree of inbreeding ($F_{IS} = -0.65$). Genetic differentiation can be represented by low values of F_{ST} between 0–0.05, moderate values between 0.05–0.15, and high values between 0.15–0.25 (Aycrigg and Garton 2014). Low genetic differentiation indicates gene flow is occurring in the population. Additionally, we calculated F_{IS} for EINPS ($F_{IS} = -0.65$) and EINPN ($F_{IS} = -0.70$) indicating low inbreeding within the 2 groups. We found low level of differentiation between EINPN–EINPS ($F_{ST} = 0.03$). All estimated 95% confidence intervals calculated using bootstrapping methods for F-statistics were significant and did not overlap 0. Individuals assigned to EINPN were more closely related than elk in EINPS and admixture groups. Average relatedness for elk within the same genetic group was determined for EINPN ($r = 0.23$), EINPS ($r = 0.03$), and for admixture ($r = 0.03$; $P < 0.007$). Average pairwise relatedness of EINPN female pairs was higher annually ($r_{xy} = 0.32$) and during the breeding season ($r_{xy} = 0.23$) than average relatedness of EINPS female pairs annually ($r_{xy} = 0.19$) and during the breeding season ($r_{xy} = 0.18$; Fig 1.5, Fig 1.6, Fig 1.7).

We used pairs identified during the same week from 9 sampling weeks from fecal samples and calculated simultaneous distances for 302 total unique elk pairs (170 female–female, 26 male–male, and 106 male–female). We found an inverse relationship between distance and relatedness ($P \leq 0.001$) and an effect of genetic group ($P \leq 0.001$) and sex ($P = 0.038$) no difference for sampling week ($P = 0.095$). The beta estimate was

91.61 for distance and -73.61 for relatedness. The beta estimate for genetic groups were -58.15 for EINPS-admixture ($P = 0.003$), -69.98 for EINPS ($P = 0.001$), and -56.92 for EINPN pairs ($P = 0.03$). Results indicated as distance increased between female pairs, relatedness decreased. For every 1 km increase in distance between pairs, relatedness decreased -38.92 for EINPN, -40.15 for EINPS-admixture, and -51.98 for EINPS.

We identified minimal spatial overlap occurring between the 2 EINP groups using 45,062 GPS locations from 29 collared elk with at least 50 GPS fixes from February 2019–December 2021 (Fig 1.3, Table 1.2) and 157 fecal samples collected on the landscape (Fig 1.4) to assess spatial distribution. Based on both collar and fecal locations, a majority of EINPS individuals were located in the western Elk Hunt Zones, with 2 EINPS individuals identified in the southwestern portion of the Sundquist unit. Elk assigned to EINPN were located in the northeastern portion of the Sundquist unit with 4 individuals in the western Elk Hunt Zones. Admixture individuals were identified across all sampled areas. Four elk had notable Kentucky influence (>10% KY assignment) with 3 identified in the Sundquist unit and 1 in the western Elk Hunt Zones.

We calculated mean 95% annual home range sizes and percentage of home range overlap in 2019, 2020, and 2021 (Table 1.3 and Table 1.4). The number of GPS fixes used for annual home range analysis ranged from 111–2094 for a total of 37,340 (Table 1.5). Mean annual 95% home range size for all elk, EINPN, EINPS, and admixture are reported in Table 1.3. Mean annual percentage of home range overlap for all pairs of sex and genetic groups are reported in Table 1.4. We found genetic group had an effect on

annual home range overlap ($P = 0.039$) but no effect of relatedness ($P = 0.404$), sex ($P = 0.198$), or year ($P = 0.162$).

We calculated mean 95% home range size and percentage of home range overlap during the breeding seasons of 2019, 2020, and 2021 (Table 1.6 and Table 1.7). Elk had at least 10 GPS fixes per month from September 1 to October 31st. The number of GPS fixes used for breeding season home range analysis ranged from 11–354 for a total of 4,792 (Table 1.8). Mean home range size for all elk, EINPN, EINPS, and admixture are represented in Table 1.6. Mean percentage of overlap for all pairs of sex and genetic groups are represented in Table 1.7. We found an effect of sex ($P = 0.019$) and genetic group ($P = 0.018$) on breeding season home range overlap but no effect of relatedness ($P = 0.620$) or year ($P = 0.334$).

DISCUSSION

Originally, a higher proportion of EINPN compared to EINPS elk were released during the first 3 years of reintroductions at the NCWMA (Muller et al. 2018). Initially, EINPN and EINPS elk were released in 3 primary areas: 2 locations west of highway I-75 (currently the western Elk Hunt Zones) and 1 location east of highway I-75 (Sundquist unit; Kindall et al. 2011). Elk from both EINP groups and admixture were released in the western Elk Hunt Zones and only EINPN and admixture were released in the Sundquist unit. Despite mixing and releasing EINP elk together during multiple releases, genetic structure has continued to persist at the NCWMA, and we determined the EINP genetic groups remain largely spatially segregated.

Previous evaluation 11+ years after reintroduction determined 41.7% (10/24) elk assigned to EINPN and 58.3% (14/24) to EINPS (Muller et al. 2018). Our results indicate the continued persistence of the 2 original EINP genetic populations used for reintroduction into Tennessee with an increase in EINPS heritage. Compared to elk originally released, we identified a lower assignment to EINPN, higher assignment to EINPS, and although not significant, an increase in admixture (15.8%), possibly indicating a decline in EINPN genetics at the NCWMA.

Reduced genetic diversity is a significant concern in isolated and reintroduced populations (Hicks et al. 2007). The risks associated with reduced genetic health can lead to negative impacts on reintroduction efforts and population viability. However, current genetic diversity ($H_e = 0.50$, 3.9 alleles/locus) increased from previous assessment of NCWMA elk by Muller et al. (2018; $H_e = 0.49$, 3.8 alleles/locus) using the same 15 microsatellites. Although direct comparisons of genetic diversity are not possible when using different microsatellite combinations, our results are similar to reports from other studies including Kentucky elk (different microsatellites than the ones used in this study; Youngmann et al. 2020) $H_e = 0.67$ with allelic richness of 3.52; Rocky Mountain elk (*C. c. nelsoni*; Hicks et al. 2007) in Yellowstone National Park populations with H_e 0.51–0.60 and allelic richness 3.32–4.04; and Tule elk (*C. c. nannodes*), Roosevelt elk (*C. c. roosevelti*), and Rocky Mountain elk in California (Meredith et al. 2007) with H_e 0.33–0.58 and mean allelic richness 3.2–6.8.

Heterozygosity and allelic richness relate to a population's ability to respond to selection and their long-term evolutionary potential (Barbosa et al. 2021). Lower

heterozygosity and allelic richness may be indicative of lower adaptive capacity and could lead to higher relatedness across individuals (Barbosa et al. 2021). For example, Tule elk show extremely low levels of heterozygosity and allelic richness as they typically remain within their respective social groups and lack group connectivity which can potentially lead to decreased gene flow (Batter et al. 2021). We observed a slight increase in H_e and allelic richness in the NCWMA population since previous evaluation (Muller et al. 2018). Minimal changes in allelic richness and genetic variation post reintroduction were also reported in a reintroduced elk population in Missouri (Pero et al. 2021). Elk at the NCWMA appear to be maintaining moderate levels of genetic diversity.

Genetic sampling during our study occurred predominately in areas across the western Elk Hunt Zones and the Sundquist unit (approximately 304.72 km²). Limited sampling areas or no viable genetic samples in the northeastern portion of the NCWMA (Ed carter unit; approximately 184.9 km²) and lower Royal Blue Unit (approximately 332.21 km²) precluded genetic evaluation in these areas. The Ed Carter unit borders the Kentucky state line and connects with the southernmost range of the Kentucky elk population. Four admixture elk identified from fecal sampling had notable proportions (>10% KY genetic assignment) of Kentucky genetic influence. Three were located in the northeastern areas of the Sundquist unit and 1 was located in the southwestern Elk Hunt Zones, indicating Kentucky influence may be occurring across the NCWMA population and contributing to sustained genetic diversity. Increasing fecal sampling effort, in conjunction with genetic monitoring in the Ed Carter unit, should be conducted to evaluate Kentucky influence and potential mixing with the NCWMA.

Pairwise F_{ST} , F_{ST} , and F_{IS} results indicated relatively low genetic differentiation and low inbreeding, similar to results found in Tule elk herds in California and restoration sites in Kentucky (Meredith et al. 2007, Youngmann et al. 2020). Low differentiation and F_{IS} have been documented in various elk populations even within geographically separated populations across a large landscape (Polziehn et al. 2000, Williams et al. 2002, Hicks et al. 2007, Aycrigg and Garton 2014, Youngmann et al. 2020). Although the 2 EINP populations are exhibiting spatial separation 20 years post reintroduction, low inbreeding, low genetic differentiation, and an increase in admixtures indicates gene flow does occur. Low F_{IS} values for both EINPN and EINPS groups and high relatedness in female pairs could be contributed to close familial relationships. Familial relationships are quantified by relatedness values designated for individual pairs, with closely related individuals having higher values ($r_{xy} = 1$ for identical pairs, $r_{xy} = 0.5$ for parents and offspring or full sibling relationships, $r_{xy} = 0.25$ for second-order relationships, and $r_{xy} = 0.125$ for third-order relationships (Lynch and Ritland 1999). While we did not test for known relative relationships, our results indicate potential sibling, cousin, parent/offspring relationships between female pairs remaining in close proximity.

Females are highly philopatric and typically remain close to their natal ranges and other closely related females (Raedeke et al. 2002). Strong site fidelity and learned behavior among females are potential contributors to consistent social group associations (Raedeke et al. 2002). We identified high genetic relatedness in female pairs identified from fecal sampling in close proximity and high proportions of annual and breeding

season home range overlap (41.02–66.08%) in collared female-female groups. Average pairwise relatedness of EINPN female pairs was higher annually ($r_{xy} = 0.32$) and during the breeding season ($r_{xy} = 0.23$) than average relatedness of EINPS female pairs annually ($r_{xy} = 0.19$) and during the breeding season (0.18; Fig 1.5, Fig 1.6, Fig 1.7). Higher r_{xy} in female pairs annually may be due in part to small sample sizes and potentially fewer familial groups represented during the breeding season. It is possible genetic structure and spatial separation between the EINP groups could be heavily influenced by strong familial relationships and site fidelity in female pairs remaining in closer proximity. Male-female pairs identified from fecal sampling were farther apart than female pairs ($P = 0.011$).

Population genetic structuring is common in ungulates and can result from factors including spatial distribution, social behavior, limited dispersal, and relatedness (Miller et al. 2010, Nobels et al. 2016). Fine-scale genetic structure was identified in desert bighorn sheep (*Ovis canadensis*) and reported as a potential outcome related to natural history, high site fidelity, skewed mating ratios, and fragmented landscapes (Jahner et al. 2018). Populations exhibiting differences in geographic distribution and little dispersal potential typically show pronounced spatial genetic structure (Carvalho et al. 2014). In herds that are not migratory, reports indicated strong site fidelity, consistent group associations, and strong social bonds between females and offspring (Raedeke et al. 2002, Sevigny et al. 2018). Our results indicate EINP elk have small annual home range sizes and positive assortative mating is occurring at the NCWMA. Genetic history could be a possible mechanism for assortative mating in wild populations, where individuals with similar

genetic qualities are more likely to breed than individuals in a random mating system (Tung et al. 2012, Day et al. 2021). While we identified low differentiation between EINPN and EINPS, positive assortative mating between elk within the same genetic group would contribute to the persistence of the 2 EINP groups at the NCWMA.

Additional population parameters including reproduction and food habits may provide valuable information and potential mechanisms also impacting the NCWMA population, if differences occur between the genetic groups. Pregnancy of adult females captured in 2019 were determined as 80% (4/5) for EINPN and 66.7% (4/6) for EINPS groups (E. Watson, University of Tennessee, unpublished data). Additionally, a minimum number of pregnant females for both EINP genetic groups was determined using female fecal samples genetically identified from landscape collection and quantifying fecal progesterone metabolite concentrations with 2 commercial Arbor Assays enzyme immunoassay kits (Ann Arbor, Michigan, USA; Progesterone EIA kit (EIA) and Progesterone Metabolite kit (PMK)). Females assigned to EINPS ($n=41$) had pregnancy percentages of 51.20% for EIA and 58.50% for PMK. Females assigned to EINPN ($n=9$) had percentages of 44.40% for EIA and 66.70% for PMK (E. Watson, University of Tennessee, unpublished data). These are minimal pregnancy rates and do not account for differences by age class. Minimal differences were identified in pregnancy percentages for EINPN and EINPS females using both kits. Further research to evaluate if differences between the EINP groups occur in subsequent reproductive parameters, including calf survival and recruitment, may help to identify possible reasons that may be contributing to the reduction in EINPN genetic assignment.

Metts (2021) did not find differences between genetic groups in winter food habits of NCWMA elk in 2019. No differences in winter food habits and small home ranges for EINP elk possibly indicate habitat and resource availability are not likely factors contributing spatial separation of the genetic groups. Although there was wide variability in winter food habits between individuals, if resources are not changing greatly over time, reinforcement of familiar sites between elk in the same genetic group, and particularly for females remaining close to their respective familial groups, could be impacting movement patterns of the 2 EINP groups (Morrison et al. 2019).

MANAGEMENT IMPLICATIONS

Through GPS collared elk, we were able to provide more insight into spatial distribution and home range with minimal overlap for the genetic groups from EINPS and EINPN than previous evaluations using VHF telemetry locations. Using simultaneous distances and pairwise relatedness from fecal samples collected on the landscape, we determined genetic relatedness increased as distance between EINP pairs decreased. Negative impacts on overall population health and viability may occur if genetic diversity is not maintained (Meredith et al. 2007, Barbosa et al. 2021). Loss of genetic diversity can lower fitness, increase susceptibility to disease, and impact annual reproductive success (Barbosa et al. 2021). However, we did not identify a decline in genetic diversity since previous evaluation during initial reintroduction. Social dynamics, positive assortative mating, and high relatedness and consistent familial groups of females within the EINP genetic groups may be contributing to the genetic structure of

the elk population. Promoting genetic diversity and population connectivity is important for maintaining overall population health of elk at the NCWMA.

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ETHICS STATEMENT

All research was approved by the University of Tennessee Institutional Animal Care and Use Committee (UT-IACUC 2671-0219).

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APPENDIX

Table 1.1 Comparison of observed (H_o) and expected (H_e) heterozygosity, observed number of alleles per locus, and deviation to Hardy-Weinberg Equilibrium (HWE) of elk at North Cumberland Wildlife Management Area in Tennessee during previous evaluation from 2000–2003 by Muller et al. (2018) and current evaluation in 2019. Locus *Rt13* was removed from current evaluation due to poor performance during Wildlife Genetics International microsatellite analysis.

Locus	GenBank Accession Number	Allelic Size Range	Previous Evaluation					Current Evaluation				
			H_o	H_e	A	N	HWE	H_o	H_e	A	N	HWE
BL42	EU009439	251.95–264.55	0.51	0.49	3	166	NS	0.35	0.41	3	101	NS
BM203	G18500	220.85–238.10	0.39	0.41	7	165	NS	0.45	0.45	7	101	NS
BM3507	G18516	147.40–168.00	0.49	0.58	4	163	NS	0.56	0.63	4	101	NS
BM4028	G18493	95.90–129.70	0.60	0.67	5	166	NS	0.50	0.52	5	101	NS
BM4107	G18519	142.30–170.20	0.56	0.55	3	167	NS	0.33	0.41	3	101	NS
BM4513	G18507	111.40–139.00	0.46	0.54	4	167	NS	0.54	0.56	5	101	NS
BM6506	G18455	196.00–218.90	0.53	0.57	3	167	NS	0.59	0.58	3	101	NS
BM888	G18484	177.50–193.10	0.62	0.64	3	167	NS	0.63	0.65	4	101	NS
BMC1009	Unknown	267.60–289.20	0.43	0.50	4	167	NS	0.60	0.59	3	101	NS
CSSM041	U03816	133.40–143.80	0.54	0.55	3	167	NS	0.51	0.52	4	101	NS
Oar	L01533	126.60–166.40	0.28	0.34	6	164	NS	0.37	0.34	5	101	NS
FCB193												
INRA107	X71577	189.80–214.96	0.49	0.52	4	166	NS	0.44	0.59	4	101	**
OvirH	L35583	107.00–125.80	0.64	0.67	4	166	NS	0.62	0.62	4	101	NS
Rt1	U90737.1	222.50–239.00	0.47	0.52	3	166	NS	0.62	0.62	3	101	NS
Rt13	U90743	311.90–333.00			1	167	ND					
Rt7	U90740	197.00–236.20	0.34	0.37	3	166	NS	0.02	0.02	2	44	ND
Mean				0.49	3.8				0.50	3.9		

^aAdapted from Bishop et al. 1994

^bSignificance with Bonferroni correction; ** = significant. NS = not significant. ND = not done (unable to analyze due to minimum expected frequency <5).

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Table 1.2. Mean ($\bar{x}$) and range of GPS fixes used to assess spatial distribution of all captured and collared elk ($n=29$) genetic groups at the North Cumberland Wildlife Management Area. Data collected from collared elk had at least 50 GPS fixes from the time of collar deployment in 2019 or 2020 through 2021.

	Male			Female			All elk		
	n	$\bar{x}$	Range	n	$\bar{x}$	Range	n	$\bar{x}$	Range
All elk	8	1302	50–3742	21	1650	62–4720	29	1554	50–4720
EINPN	2	403	50–756	7	2606	1010–4720	9	2116	50–4720
EINPS	5	1311	144–3742	13	1088	62–2918	18	1150	62–3742
admixture	1	3052	N/A	1	2260	N/A	2	2656	2260–3052

Table 1.3. Mean ($\bar{x}$) $\pm$ standard error (SE) annual 95% home range size (km²) for male and female elk in 2019 ($n=3$), 2020 ($n=23$), and 2021 ($n=14$) at the North Cumberland Wildlife Management Area in Tennessee using GPS fixes collected from collared elk. We included elk with at least 100 GPS points from the time of collar deployment through last calendar day of the year (December 31st) or from January 1 through December 31st. Home range size was calculated using the kernel density tool in the Spatial Analysis package in ArcMap.

	Year	n	Male		n	Female	
			$\bar{x} \pm \text{SE}$	Range		$\bar{x} \pm \text{SE}$	Range
All elk	2019	3	1.10 $\pm$ 0.47	0.1–1.73	10	3.29 $\pm$ 0.55	0.45–5.89
	2020	6	5.00 $\pm$ 1.97	0.04–10.44	17	5.27 $\pm$ 0.07	0.05–11.55
	2021	2	14.35 $\pm$ 14.08	0.27– 28.42	12	5.10 $\pm$ 1.03	0.02–11.84
EINPN	2019	1	1.73	N/A	5	2.30 $\pm$ 0.53	0.45–3.16
	2020	2	1.28 $\pm$ 0.32	0.96–1.59	7	2.93 $\pm$ 0.26	2.10–4.14
	2021	0	0	0	6	2.66 $\pm$ 0.50	0.75–4.58
EINPS	2019	2	0.47 $\pm$ 0.37	0.1–0.84	5	4.27 $\pm$ 0.78	1.82–5.89
	2020	3	3.18 $\pm$ 3.07	0.04–9.32	9	3.61 $\pm$ 1.16	0.05–11.55
	2021	1	0.27	N/A	5	3.35 $\pm$ 2.19	0.02–11.84
admixture	2019	0	0	0	0	0	0
	2020	1	10.55	N/A	1	9.27	N/A
	2021	1	28.42	N/A	1	9.29	N/A

Table 1.4. Mean ($\bar{x}$) $\pm$ standard error (SE) percentage of annual 95% home range overlap for male and female elk in 2019 ($n=30$), 2020 ($n=80$), and 2021 ($n=28$) at the North Cumberland Wildlife Management Area in Tennessee. Data were collected from collared elk with at least 100 GPS points from the time of collar deployment through last calendar day of the year (December 31st) or from January 1st to December 31st. Home range size was calculated using the kernel density tool in the Spatial Analysis package in ArcMap. Percentage of home range overlap was determined by calculating overlap area of 2 home ranges, dividing by mean home range size of each individual, and multiplying by 100.

	Year	<i>n</i>	female-female		<i>n</i>	male-female		<i>n</i>	male-male	
			$\bar{x} \pm \text{SE}$	Range		$\bar{x} \pm \text{SE}$	Range		$\bar{x} \pm \text{SE}$	Range
All elk	2019	16	66.08 $\pm$ 6.30	19.65–100.00	13	19.16 $\pm$ 5.73	0.79–50.69	1	3.88	N/A
	2020	41	54.26 $\pm$ 4.52	2.41–105.85	34	25.28 $\pm$ 4.16	0.74–90.34	5	17.11 $\pm$ 8.91	0.91–42.96
	2021	21	47.35 $\pm$ 6.00	1.02–95.70	6	12.05 $\pm$ 9.78	0.01–65.51	1	2.13	N/A
EINPN-EINPN	2019	10	56.58 $\pm$ 8.73	19.65–100.00	5	43.28 $\pm$ 3.83	28.71–50.69	0	0	N/A
	2020	21	65.59 $\pm$ 4.32	40.02–93.45	7	22.04 $\pm$ 1.93	12.11–26.89	0	0	N/A
	2021	15	52.32 $\pm$ 4.66	27.52–92.51	0	0	N/A	0	0	N/A
EINPS-EINPS	2019	6	75.59 $\pm$ 6.62	55.20–97.34	8	4.08 $\pm$ 1.40	0.79–12.21	1	3.88	N/A
	2020	17	36.46 $\pm$ 7.62	2.42–100.00	14	26.27 $\pm$ 7.73	0.74–83.28	1	42.96	N/A
	2021	5	22.77 $\pm$ 17.71	0.48–93.10	2	41.60 $\pm$ 23.90	17.70–65.51	0	0	N/A
EINPN-EINPS	2019	0	0	N/A	0	0	N/A	0	0	N/A
	2020	0	0	N/A	6	13.17 $\pm$ 2.76	5.48–23.25	1	3.53	N/A
	2021	0	0	N/A	0	0	N/A	0	0	N/A
admix-admix	2019	0	0	N/A	0	0	N/A	0	0	N/A
	2020	0	0	N/A	0	0	N/A	0	0	N/A

Table 1.4
continued

	Year	female-female			male-female			male-male		
		<i>n</i>	$\bar{x} \pm \text{SE}$	Range	<i>n</i>	$\bar{x} \pm \text{SE}$	Range	<i>n</i>	$\bar{x} \pm \text{SE}$	Range
	2021	0	0	N/A	0	0	N/A	0	0	N/A
admix-EINPN	2019	0	0	N/A	0	0	N/A	0	0	N/A
	2020	0	0	N/A	0	0	N/A	1	34.34	N/A
	2021	0	0	N/A	0	0	N/A	0	0	N/A
admix-EINPS	2019	0	0	N/A	0	0	N/A	0	0	N/A
	2020	3	75.83 $\pm$ 8.26	63.74–91.62	7	36.90 $\pm$ 12.15	1.13–90.34	2	2.36 $\pm$ 1.45	0.91–3.81
	2021	1	95.79	N/A	4	10.64 $\pm$ 6.11	0.01–25.70	1	2.14	N/A

Table 1.5. Mean ($\bar{x}$) and range of GPS fixes from captured and collared elk in 2019 ($n=13$), 2020 ($n=23$), and 2021($n=14$) used for estimating annual 95% home range sizes at the North Cumberland Wildlife Management Area in Tennessee. Elk had at least 100 GPS fixes from the time of collar deployment until the last day of the calendar year (December 31st) or annually from January 1st to December 31^s.

	Year	n	Male		n	Female		n	Total	
			$\bar{x}$	Range		$\bar{x}$	Range		$\bar{x}$	Range
All elk	2019	3	284	154–353	10	291	111–455	13	289	111–455
	2020	6	839	285–1807	17	880	192–2094	23	869	192–2094
	2021	2	1153	785–1521	12	940	115–2085	14	971	115–2085
EINPN	2019	1	N/A	N/A	5	328	185–455	6	331	185–455
	2020	2	200	118–285	7	1139	373–2094	9	930	118–2094
	2021	0	0	0	6	1052	212–2085	6	1052	212–2085
EINPS	2019	2	254	154–353	5	255	111–350	7	254	111–353
	2020	3	1043	394–1807	9	702	192–1422	12	768	192–1807
	2021	1	1521	N/A	5	776	115–1350	6	900	115–1521
admixture	2019	0	0	0	0	0	0	0	0	0
	2020	1	1501	N/A	1	899	N/A	2	1200	899–1501
	2021	1	785	N/A	1	1097	N/A	2	941	785–1097

Table 1.6. Mean ($\bar{x}$) $\pm$ standard error (SE) of 95% home range size (km²) for male and female elk during the breeding season of 2019 ($n=10$), 2020 ($n=17$), and 2021 ($n=7$) at the North Cumberland Wildlife Management Area in Tennessee using GPS fixes collected from collared elk. Data were collected from collared elk with at least 10 GPS per month from September 1st to October 31st. Home range size was calculated using the kernel density tool in the Spatial Analysis package in ArcMap.

	Year	n	Male		n	Female	
			$\bar{x} \pm \text{SE}$	Range		$\bar{x} \pm \text{SE}$	Range
All elk	2019	3	0.30 $\pm$ 0.23	0.01–0.75	7	2.55 $\pm$ 0.23	1.58–3.37
	2020	5	3.23 $\pm$ 1.72	0.03–8.93	12	2.24 $\pm$ 0.35	0.01–3.92
	2021	1	0.1	N/A	6	2.56 $\pm$ 0.51	0.35–3.78
EINPN	2019	1	0.15	N/A	3	2.05 $\pm$ 0.24	1.58–2.26
	2020	2	0.71 $\pm$ 0.18	0.53–0.88	5	1.46 $\pm$ 0.14	0.98–1.78
	2021	0	0	N/A	3	1.11 $\pm$ 0.38	0.35–1.56
EINPS	2019	2	0.38 $\pm$ 0.37	0.01–0.75	4	3.05 $\pm$ 0.11	2.92–3.37
	2020	2	0.07 $\pm$ 0.04	0.03–0.10	6	1.97 $\pm$ 0.66	0.01–3.92
	2021	1	0.1	N/A	2	2.78 $\pm$ 0.43	2.34–3.21
admixture	2019	0	0	N/A	0	0	N/A
	2020	1	8.93	N/A	1	3.29	N/A
	2021	0	0	N/A	1	3.78	N/A

Table 1.7. Mean ($\bar{x}$) $\pm$ standard error (SE) percentage of 95% home range overlap for elk during the breeding season of 2019 ($n=11$), 2020 ($n=36$), and 2021 ($n=6$) at the North Cumberland Wildlife Management Area in Tennessee. Data were collected from collared elk with at least 10 GPS per month from September 1st to October 31st. Home range size was calculated using the kernel density tool in the Spatial Analysis package in ArcMap. Percentage of home range overlap was determined by calculating overlap area of 2 home ranges, dividing by mean home range size of each individual, and multiplying by 100.

	Year	<i>n</i>	female-female		<i>n</i>	male-female		<i>n</i>	male-male	
			$\bar{x} \pm \text{SE}$	Range		$\bar{x} \pm \text{SE}$	Range		$\bar{x} \pm \text{SE}$	Range
All elk	2019	6	63.82 $\pm$ 12.24	26.90–94.84	5	0.90 $\pm$ 0.01	0.87–0.92	0	0	0
	2020	16	41.02 $\pm$ 7.00	1.29–83.45	14	17.55 $\pm$ 5.56	0.42–57.49	6	17.84 $\pm$ 8.26	0.90–55.29
	2021	4	42.25 $\pm$ 17.72	5.84–89.21	1	9.83	N/A	0	0	0
EINPN-EINPN	2019	3	47.36 $\pm$ 19.39	26.90–86.12	1	0.87	N/A	0	0	0
	2020	9	44.96 $\pm$ 7.93	8.02–76.64	1	3.66	N/A	0	0	0
	2021	3	26.59 $\pm$ 11.74	5.84–46.48	0	0	0	0	0	0
EINPS-EINPS	2019	3	80.28 $\pm$ 10.11	60.85–94.84	4	0.91 $\pm$ 0.01	0.87–0.92	0	0	0
	2020	5	25.21 $\pm$ 15.48	1.29–83.45	5	6.32 $\pm$ 1.92	2.05–12.65	1	55.29	N/A
	2021	0	0	0	1	9.83	N/A	0	0	0
EINPN-EINPS	2019	0	0	0	0	0	0	0	0	0
	2020	0	0	0	4	23.28 $\pm$ 8.63	4.18–45.16	2	12.55 $\pm$ 3.66	8.90–16.21
	2021	0	0	0	0	0	0	0	0	0
admix-admix	2019	0	0	0	0	0	0	0	0	0
	2020	0	0	0	0	0	0	0	0	0
	2021	0	0	0	0	0	0	0	0	0

Table 1.7 continued

	Year	female-female			male-female			male-male		
		<i>n</i>	$\bar{x} \pm \text{SE}$	Range	<i>n</i>	$\bar{x} \pm \text{SE}$	Range	<i>n</i>	$\bar{x} \pm \text{SE}$	Range
admix-EINPN	2019	0	0	0		0	0	0	0	0
	2020	0	0	0	0	0	0	1	23.45	N/A
	2021	0	0	0	0	0	0	0	0	0
admix-EINPS	2019	0	0	0	0	0	0	0	0	0
	2020	2	62.79 $\pm$ 6.70	56.09–69.80	4	29.31 $\pm$ 16.22	0.42–57.49	2	1.6 $\pm$ 0.70	0.90–2.30
	2021	1	89.21	N/A	0	0	0	0	0	0

Table 1.8. Mean ($\bar{x}$) and range of GPS fixes from captured and collared elk in 2019 ($n=10$), 2020 ($n=17$), and 2021 ($n=7$) used for estimating 95% home range size during the breeding season from September 1st to October 31st at the North Cumberland Wildlife Management Area in Tennessee. Elk had at least 10 GPS points per month.

	Year	Male			Female			Total		
		n	$\bar{x}$	Range	n	$\bar{x}$	Range	n	$\bar{x}$	Range
All elk	2019	3	41	20–61	7	64	30–80	10	58	20–80
	2020	5	123	11–279	12	174	24–354	17	159	11–354
	2021	1	279	N/A	6	228	120–354	7	235	120–354
EINPN	2019	1	43	N/A	3	77	74–80	4	69	43–80
	2020	2	31	11–50	5	229	67–354	7	172	11–354
	2021	0	N/A	N/A	3	290	176–354	3	290	176–354
EINPS	2019	2	41	20–61	4	55	30–66	6	50	20–66
	2020	2	165	51–279	6	135	24–238	8	143	24–279
	2021	1	279	N/A	2	189	178–199	3	219	178–279
admix	2019	0	N/A	N/A	0	N/A	N/A	0	N/A	N/A
	2020	1	226	N/A	1	132	N/A	2	179	132–266
	2021	0	N/A	N/A	1	120	N/A	1	120	N/A

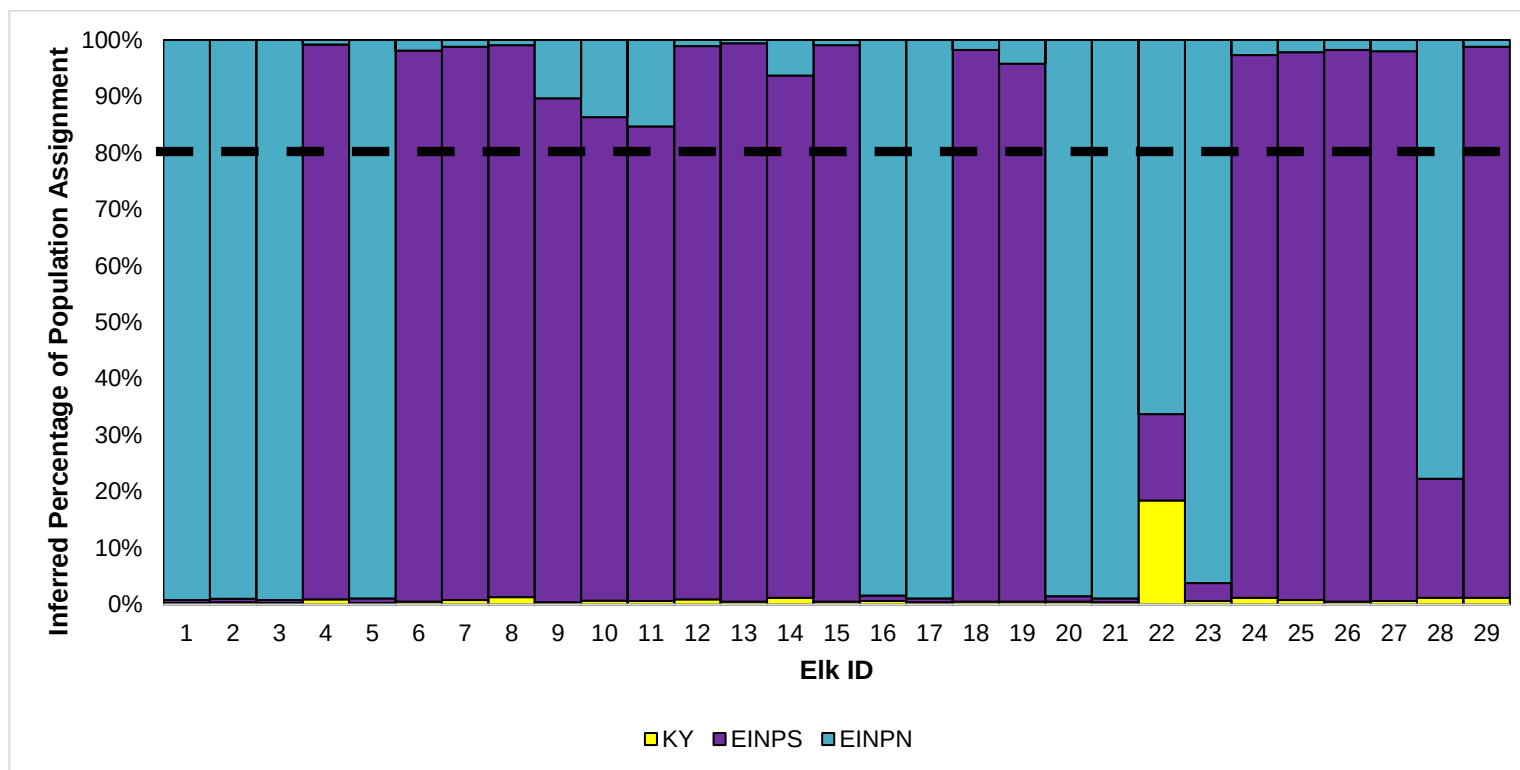


Figure 1.1. STRUCTURE results using a 3 population assumption from hair samples collected from anesthetized collared elk ($n=29$) at the North Cumberland Wildlife Management Area in Tennessee during 2019 and 2020. The dotted line at 80% represents the inferred percentage of population membership assignment used to designate individuals to 1 genetic population. Elk with <80% population membership assignment to 1 population are designated as admixture. Blue represents Elk Island National Park North (EINPN), purple represents Elk Island National Park South (EINPS), and yellow represents Kentucky. Nine elk were assigned to EINPN, 18 to EINPS, and 3 to admixture of all genetic populations.

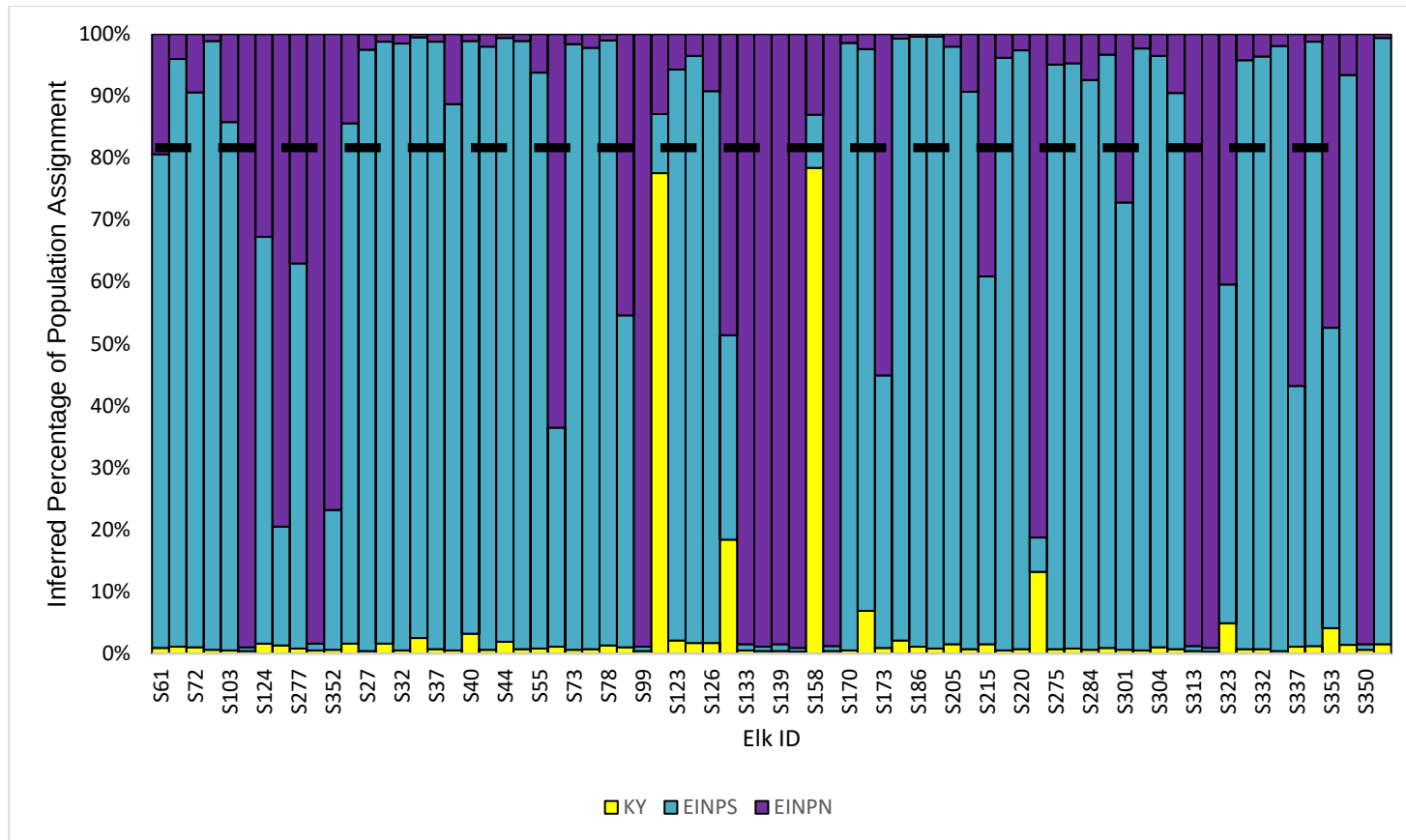


Figure 1.2. STRUCTURE results of population assignment using a 3 population assumption for elk genetically identified from fecal samples collected on the landscape in 2019 at the North Cumberland Wildlife Management Area in Tennessee. The dotted line at 80% represents the inferred percentage of population assignment used to designate individuals to 1 population. Blue represents Elk Island National Park North (EINPN), purple represents Elk Island National Park South (EINPS), and yellow represents Kentucky. Twelve elk were assigned to EINPN, 46 to EINPS, and 14 to admixture of all genetic populations.

Figure 1.3. Spatial distribution of the genetic groups from GPS locations of collared elk ($n=29$) at the North Cumberland Wildlife Management Area in Tennessee, (February 2019–December 2021) based upon hair DNA analysis. Genetic populations were determined based upon an $\geq 80\%$ assignment to 1 population using a 3 population assumption including data from known Kentucky elk ($n=295$) in program STRUCTURE. Purple represents elk assigned to Elk Island National Park South (EINPS, $n=18$), blue assigned to Elk Island National Park North (EINPN, $n=9$), and red assigned to admixture ($n=2$; 17.3% KY 59.1% EINPN 23.6% EINPS, 1.8% KY 62% EINPN 36.3% EINPS).

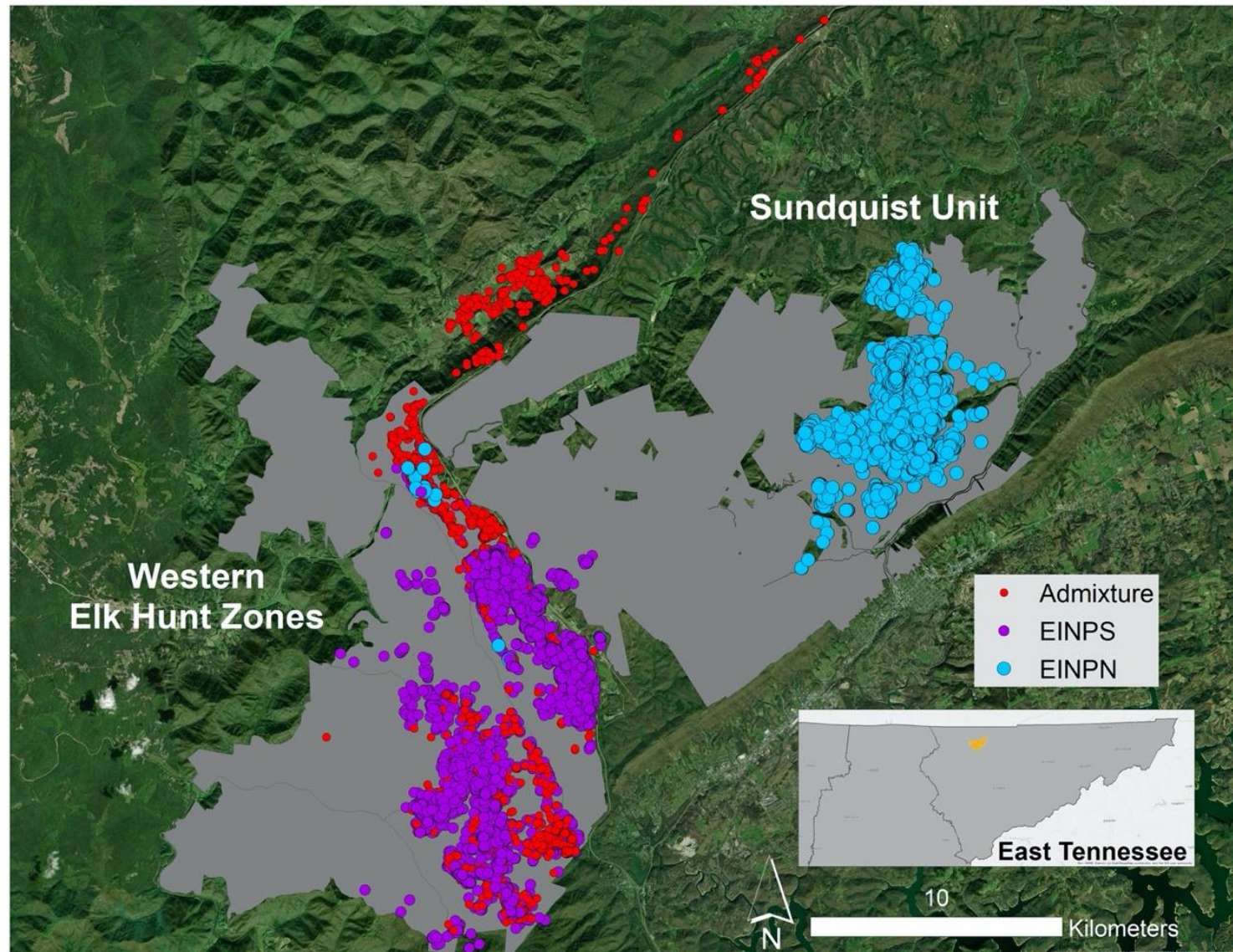


Figure 1.4. Spatial distribution of elk ($n=72$) genetically identified through elk DNA from feces collected during the 2019 landscape collection at the North Cumberland Wildlife Management Area (NCWMA) in Tennessee. Elk locations are represented by GPS coordinates recorded upon sample collection of 65 areas across the western Elk Hunt Zones and Sundquist unit of the NCWMA. Only first locations were used for elk with multiple samples collected. Genetic populations were determined based on an $\geq 80\%$ assignment to 1 population using a 3 population assumption including data from known Kentucky elk ($n=295$) in the program STRUCTURE. Purple represents elk assigned to Elk Island National Park South (EINPS, $n=46$), blue represents Elk Island National Park North (EINPN, $n=12$), and admixture ($n=14$) in red. Four elk had $>10\%$ Kentucky influence and were located in the northeastern area of the Sundquist unit ($n=3$) and southwestern Elk Hunt Zones ($n=1$).

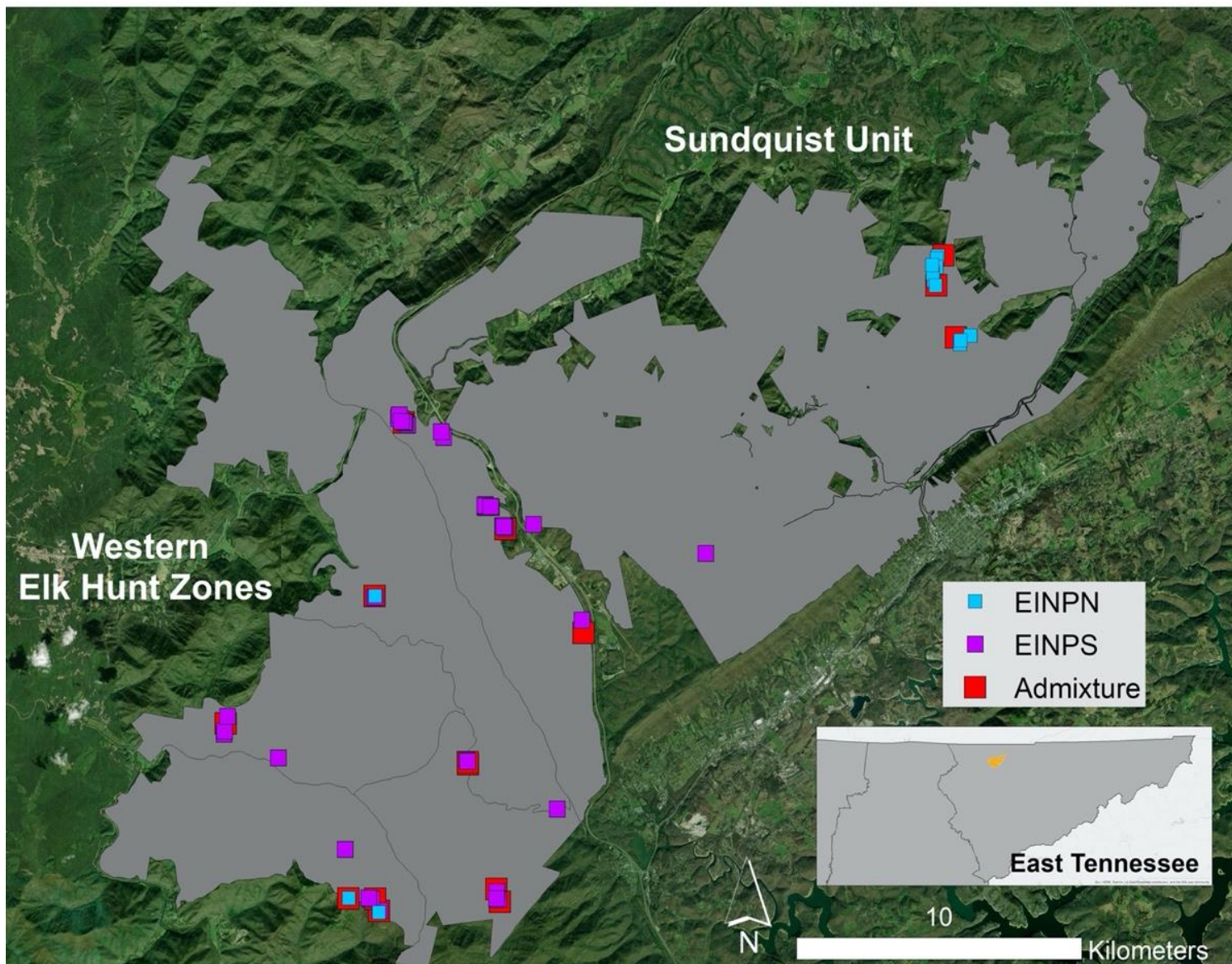
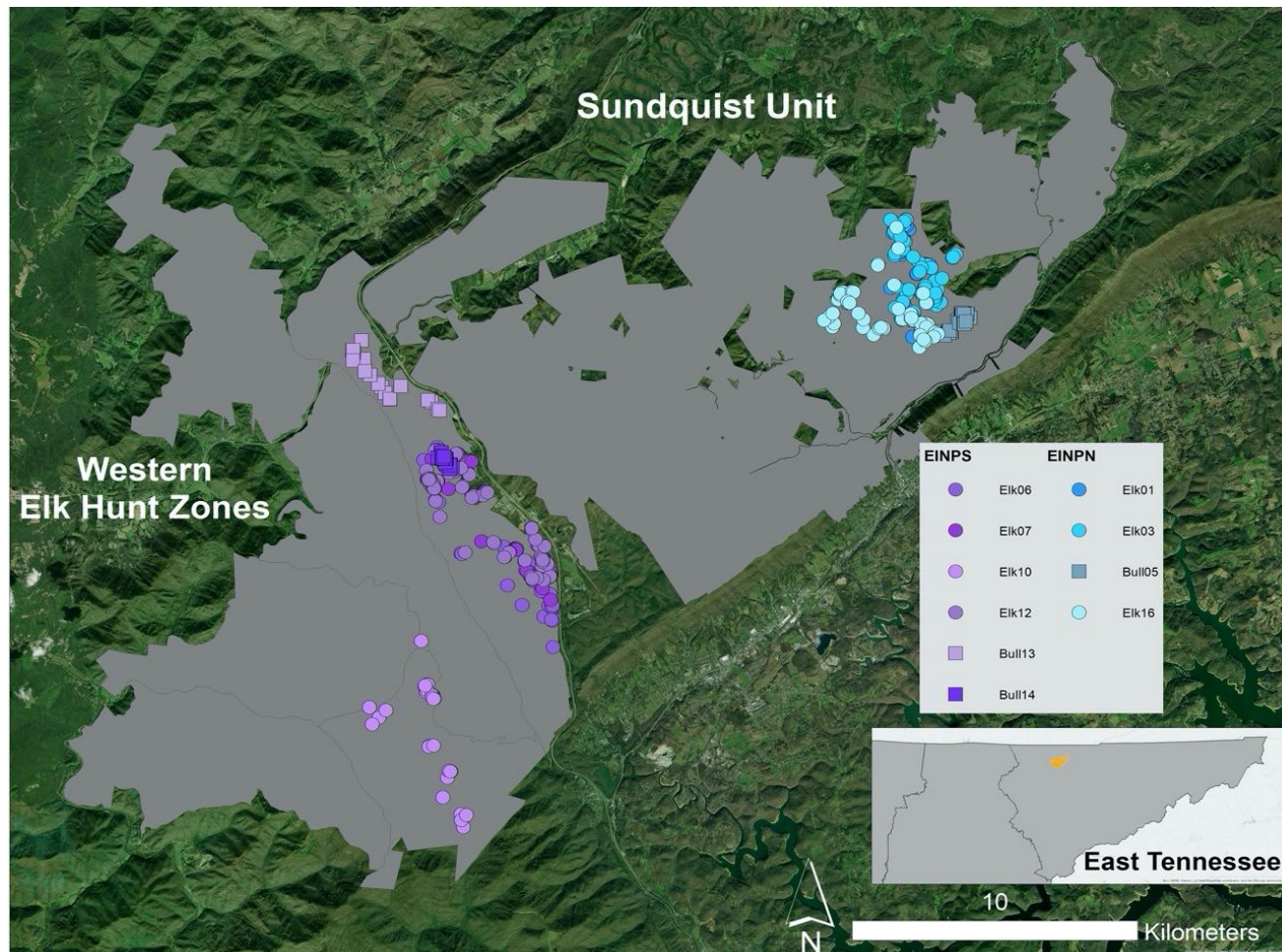
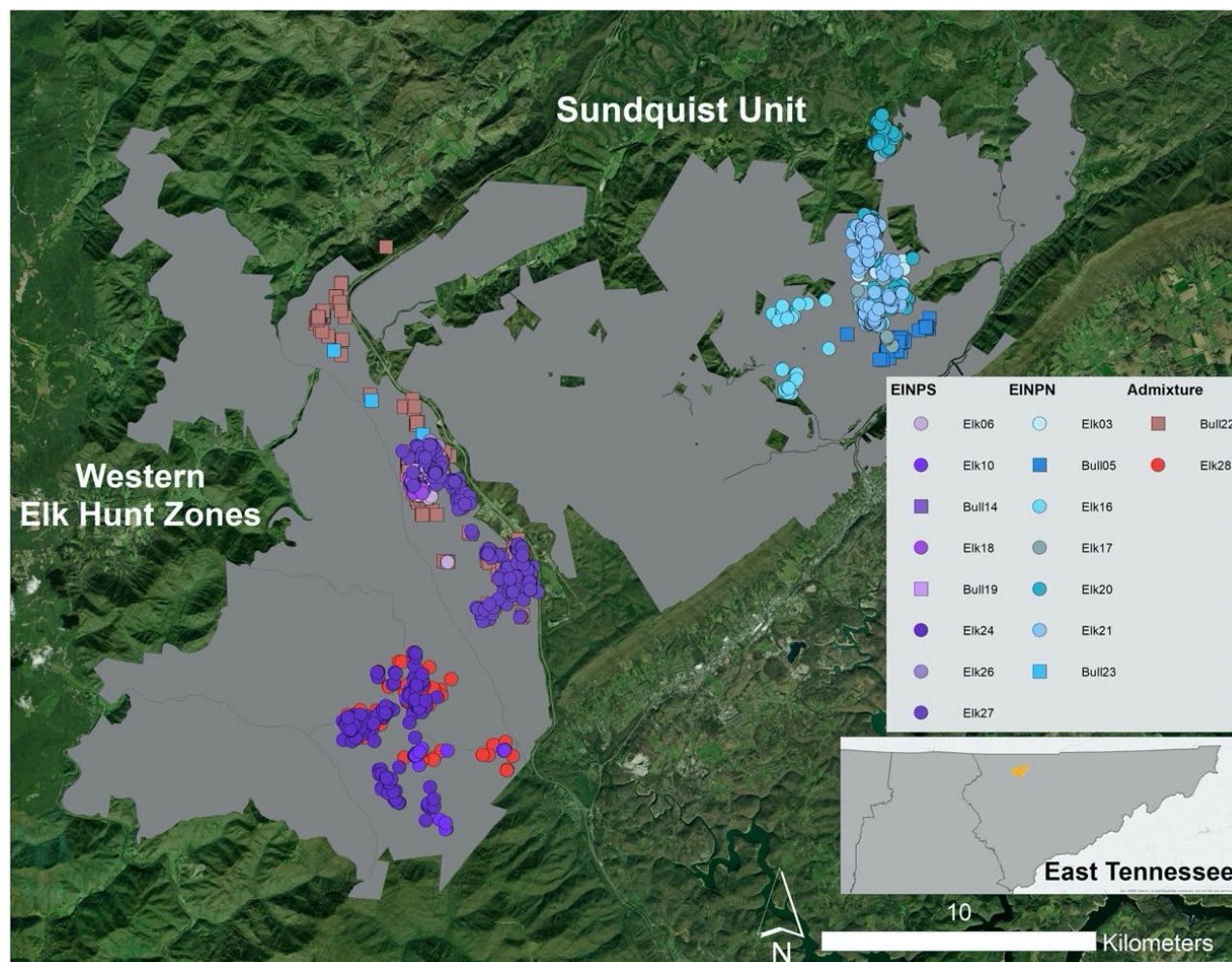


Figure 1.5. Spatial distribution of the genetic groups from GPS locations of collared elk during the 2019 breeding season at the North Cumberland Wildlife Management Area in Tennessee, based upon hair DNA analysis. Genetic populations were determined based upon an $\geq 80\%$ assignment to 1 population and a 3 population assumption including data from known Kentucky elk ($n=295$) in program STRUCTURE. Males are represented as squares and females are represented as circles. Elk Island National Park South elk (EINPS) are represented in purple ($n=6$) and Elk Island National Park North (EINPN) in blue ($n=4$). Relatedness (r_{xy}) of female pairs during the breeding season are represented on the right.



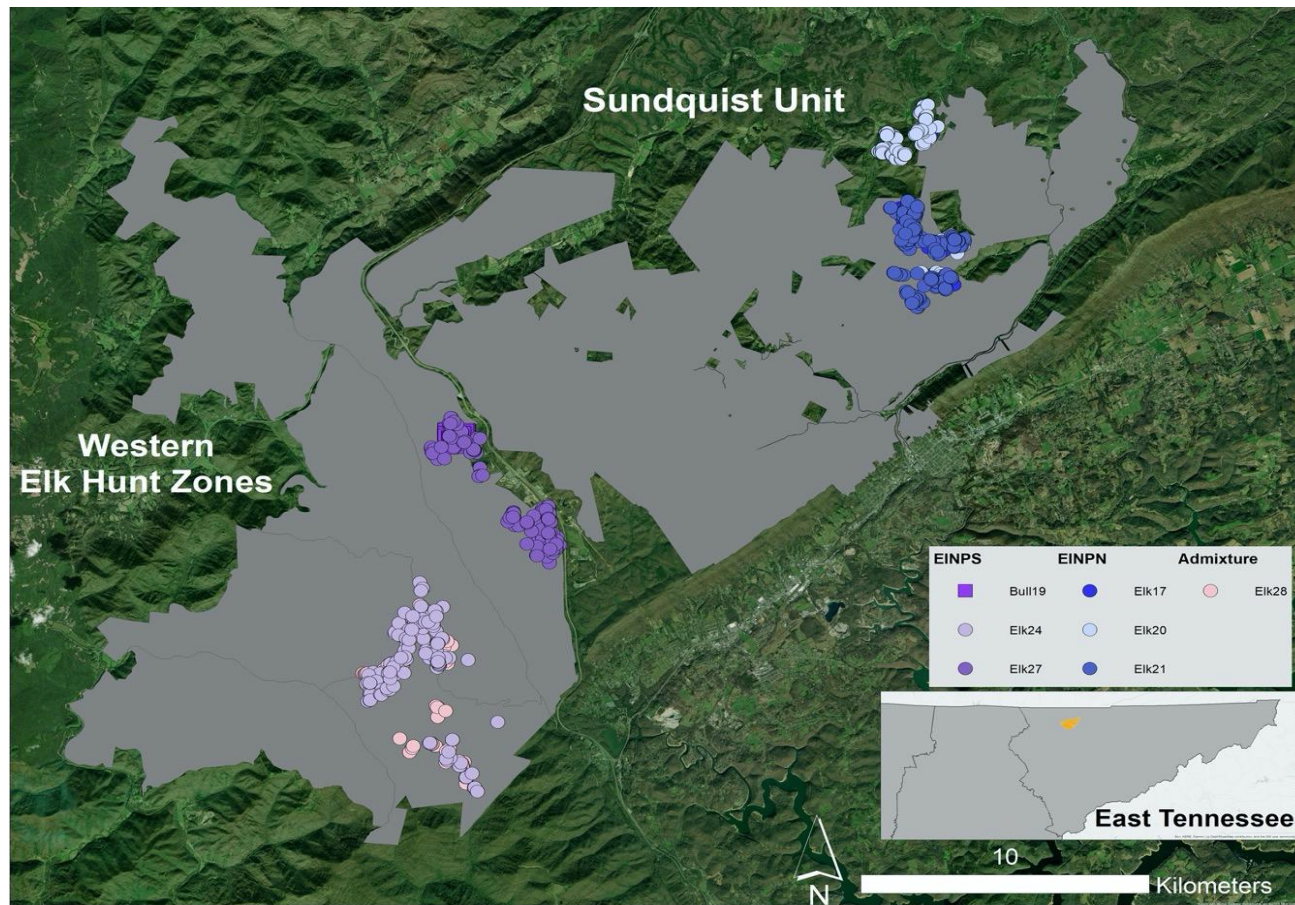
Elk 1	Elk 2	r_{xy}
Elk06	Elk07	0.27
Elk06	Elk10	0.21
Elk06	Elk12	-0.23
Elk07	Elk10	-0.09
Elk07	Elk12	-0.25
Elk10	Elk12	-0.04
Elk01	Elk03	0.53
Elk01	Elk16	0.19

Figure 1.6. Spatial distribution of the genetic groups from GPS locations of collared elk during the 2020 breeding season at the North Cumberland Wildlife Management Area in Tennessee, based upon hair DNA analysis. Genetic populations were determined based upon an $\geq 80\%$ assignment to 1 population and a 3 population assumption including data from known Kentucky elk ($n=295$) in program STRUCTURE. Males are represented as squares and females are represented as circles. Elk Island National Park South (EINPS) elk are represented in purple ($n=8$), Elk Island National Park North (EINPN) in blue ($n=7$), and admixture in red ($n=2$). Relatedness (r_{xy}) of female pairs during the breeding season are represented on the right.



Elk 1	Elk 2	r_{xy}
Elk10	Elk24	0.36
Elk16	Elk17	0.26
Elk16	Elk21	0.29
Elk16	Elk03	0.28
Elk17	Elk20	-0.02
Elk17	Elk21	0.24
Elk17	Elk03	0.20
Elk18	Elk06	0.45
Elk20	Elk21	0.19
Elk20	Elk03	0.48
Elk21	Elk03	0.18
Elk26	Elk27	-0.02
Elk26	Elk06	-0.02
Elk27	Elk06	0.82
Elk28	Elk10	0.42
Elk28	Elk24	-0.11

Figure 1.7. Spatial distribution of the genetic groups from GPS locations of collared elk during the 2021 breeding season at the North Cumberland Wildlife Management Area in Tennessee, based upon hair DNA analysis. Genetic populations were determined based upon an $\geq 80\%$ assignment to 1 population and a 3 population assumption including data from known Kentucky elk ($n=295$) in program STRUCTURE. Males are represented as squares and females are represented as circles. Elk Island National Park South (EINPS) elk are represented in purple ($n=4$), Elk Island National Park North (EINPN) in blue ($n=2$), and admixture in red ($n=1$). Relatedness (r_{xy}) of female pairs during the breeding season are represented on the right.



Elk 1	Elk 2	r_{xy}
Elk24	Elk27	0.04
Elk17	Elk20	-0.02
Elk17	Elk21	0.24
Elk20	Elk21	0.19

CHAPTER II
EVALUATING THE EFFICACY OF NON-INVASIVE FECAL
SAMPLING FOR PREGNANCY DETECTION IN ELK

A version of this chapter was written and submitted for publication in the Journal of Wildlife Diseases by Eryn M. Watson with co-authors Katherine A. Kurth, Dailee L. Metts, Sarah E. Moorey, Brad F. Miller, Richard W. Gerhold, and Lisa I. Muller.

Eryn M. Watson collected and analyzed data and wrote the article. Katherine A. Kurth and Dailee L. Metts assisted with data collection. Sarah E. Moorey, Brad F. Miller, Richard W. Gerhold, and Lisa I. Muller assisted with manuscript preparation.

ABSTRACT

Elk (*Cervus canadensis*) were reintroduced to Tennessee in the early 2000s with limited reproductive monitoring since initial release. We assessed the efficacy of non-invasive sampling for determining pregnancy status using invasive (capture) and non-invasive (fecal collection in the field) techniques at the North Cumberland Wildlife Management Area (NCWMA). We used pregnancy-specific protein B (PSPB) in blood to determine pregnancy and compared results to fecal progesterone metabolite (FPM) concentrations collected from 20 females captured during 2019 and 2020. From February to May 2019, we collected 357 fecal samples in 65 areas across 489.62 km² of the NCWMA. We identified individuals using extracted DNA and analysis of 15 microsatellites using hair from captured elk and feces collected on the landscape. We identified 62 unique individuals from 128 female fecal samples. Using Arbor Assays Progesterone enzyme immunoassay (EIA) and Progesterone Metabolite kits, we determined FPM concentrations of captured females and established FPM concentration reference range to

categorize pregnancy from feces collected non-invasively. Using PSPB concentrations, 8/11 and 3/4 of captured adult elk (≥ 2.5 years of age) were pregnant in 2019 and 2020, respectively. No 1.5-year-old captured elk were pregnant ($n=5$). Using the Progesterone EIA kit, FPM concentrations were $\bar{x}=192.84 \pm 38.63$ ng/g (95% CI, 96.48–289.20) for non-pregnant and $\bar{x}=536.17 \pm 74.98$ ng/g (95% CI, 375.97–696.36) for pregnant captured females. For the Progesterone Metabolite kit, FPM concentrations were $\bar{x}=188.16 \pm 43.39$ ng/g (95% CI, 76.63–299.69) for non-pregnant and $\bar{x}=693.52 \pm 126.52$ ng/g (95% CI, 407.31–979.72) for pregnant captured females. We categorized females from non-invasive collection as non-pregnant, undetermined, or pregnant based on a 95% CI of captured female FPM concentrations. We were unable to distinguish age classes therefore pregnancy numbers include younger age classes not expected to be pregnant. Analysis of FPM can be applied on a population level to detect pregnancy.

INTRODUCTION

Determining pregnancy is important for evaluating the health and growth potential of wild animal populations. Pregnancy-specific protein B (PSPB) is a protein hormone found in ruminant species that is secreted by the placenta and can be used to determine pregnancy with high accuracy in many species, including elk (*Cervus canadensis*; Noyes et al. 1997; Seixas et al. 2019) and white-tailed deer (*Odocoileus virginianus*; Osborn et al. 1995). However, PSPB must be measured from a blood sample requiring invasive capture. Thus, PSPB testing for large-scale population evaluation may be difficult and impractical.

Reliable methods to measure steroid hormone concentrations associated with reproduction also allow for monitoring individuals and reproductive efforts (Graham et al. 2001; Kersey and Dehnhard 2014). Circulating free steroid hormone concentrations such as progesterone and estradiol have been reported as accurate representations of gonadal function and can be measured to evaluate seasonal hormone profiles, diagnose pregnancy, and monitor basic reproductive physiology of an individual (Schwarzenberger et al. 1996; Graham et al. 2001). Progesterone is a 21 carbon, progestin steroid hormone present in a variety of tissues within the body and is produced by the corpus luteum after ovulation, the corpus luteum and placenta during pregnancy, and the adrenal gland (Plotka et al. 1982; Taraborrelli 2015; Litwack 2018). In elk, progesterone levels increase after conception and remain high throughout gestation before declining shortly before parturition (Hudson and Haigh 2002). The most accurate method for measuring true concentrations of circulating steroid hormones is through a blood sample (Touma and Palme 2005; Peter et al. 2018). However, in white-tailed deer, high concentrations of serum progesterone were excreted by the adrenal gland during capture events (Plotka et al. 1982). Stress during capture may affect serum progesterone concentrations and may lead to misidentification of pregnancy status (Hollenstein et al 2006; Togashi et al. 2009).

Measuring serum hormone concentrations on large spatial and temporal scales from wild, free-ranging animals is not always feasible (Borque et al. 2011). Invasive methods require chemical immobilization or physical restraint of an animal, which can influence circulating hormone concentrations and alter the endocrine profile of an individual (Messier et al. 1990; Cain et al. 2012). Therefore, non-invasive techniques

such as collecting fecal samples on the landscape have become increasingly popular for monitoring reproductive parameters in wild, free-ranging populations. Non-invasive techniques are routinely used as a viable alternative for steroid hormone monitoring in wild species as metabolites are relatively stable in feces and allow for more frequent collections of samples unaffected by invasive sampling stress (Kumar et al. 2013).

Steroid hormones are well suited for measurement from non-invasively collected samples. Steroids are predominately metabolized by the liver and excreted in urine or feces. Concentrations of hormones can be quantified in both urine and feces through a variety of metabolites (Schwarzenberger et al. 1996). Feces is a practical sample as it is easily obtained and can be collected across a landscape or gestational period.

Concentrations of progesterone metabolites in feces show similar patterns to plasma values and have been used to monitor pregnancy (Schwarzenberger et al. 1997). Non-invasive techniques to assess fecal progesterin, free progesterone, or progesterone metabolite concentrations for determining pregnancy status have been previously evaluated and validated in elk (Garrott et al. 1995; White et al. 1995) and other ungulate species (Morden et al. 2011; Cain et al. 2012; Krepschi et al. 2013; Wang et al. 2016). Non-invasive techniques for measuring fecal progesterone metabolites (FPM) concentrations in elk may provide an ability to sample large areas, across gestational periods, and without potentially confounding stress hormones to provide the best method for evaluating pregnancy status of females for population level assessment.

Elk were reintroduced to the North Cumberland Wildlife Management Area (NCWMA) beginning in the early 2000s and have since established a population in East

Tennessee. Detailed information on reintroduction was described in Kindall et al. (2011). Pregnancy status of adult females released during the initial 3 years of reintroduction were determined as 57.7%, 73.7%, and 93.3%, respectively (Muller et al. 2004). Pregnancy status has not been reported for the population since initial reintroduction. Updated information on elk pregnancy at the NCWMA is needed to assess population growth potential.

The objectives of our study were to compare FPM concentrations of feces from female elk collected at the time of capture with PSPB (standard test for pregnancy determination), compare 2 commercial progesterone enzyme immunoassay (EIA) kits for FPM analysis, and evaluate the efficacy of non-invasive fecal sampling for determining pregnancy status on a population scale. Evaluating the use of non-invasive fecal sampling for measuring FPM concentrations on a population scale may provide managers an alternative, valuable method for assessing pregnancy.

MATERIALS AND METHODS

Study Area

In 2000, Tennessee Wildlife Resources Agency (TWRA) established a 271,139 ha Elk Restoration Zone for elk reintroduction (TWRA 2000). We conducted research at the NCWMA located within the Elk Restoration Zone. The NCWMA is a mixed-mesophytic forest comprised of 79,318 ha of public and leased private land managed by TWRA (TWRA 2018). The NCWMA is approximately 86% deciduous forest, 12% wildlife openings (grasslands, fields, and reclaimed coal strip mines), and 1% cropland (TWRA 2018).

Elk Capture and Sample Collection

All research was approved by the University of Tennessee Institutional Animal Care and Use Committee (UT-IACUC 2671-0219). During the winters of 2019 and 2020, we captured 29 elk (21 females and 8 males) at the NCWMA using corral traps and free darting with chemical immobilization. Elk were anesthetized with an intramuscular (IM) injection of 2 mL (females and small males) and 3 mL (large males) of BAM (Each 1 mL of BAM is comprised of 27.3 mg of butorphanol, 9.1 mg of azaperone, and 10.9 mg of medetomidine; ZooPharm, Windsor, Colorado, USA) administered by darts (PneuDart Inc., Williamsport, Pennsylvania) and a Dan-Inject CO2 Rifle (Dan-Inject, Kolding, Denmark). Elk were continuously monitored under anesthesia and vitals were recorded every 5–10 minutes. Supplemental oxygen was administered intranasally if oxygen saturation fell below 90%. We recorded sex and age and fitted elk with GPS collars for concurrent studies. Samples collected from each elk included whole blood from the jugular or cephalic vein stored in vacutainers (BD, Franklin Lakes, New Jersey), hair, and a fecal sample. We separated blood samples into 2 mL increments and stored in microcentrifuge tubes at -20° C until analysis. We reversed elk immobilization with an IM injection of 4 mL (females and small males) and 6 mL (large males) of atipamezole (25 mg/mL; ZooPharm) and 0.5 mL (both males and females) of naltrexone (50 mg/mL; ZooPharm) and monitored elk until fully recovered.

Non-invasive Landscape Fecal Collection

Over a 10-week period from February to April 2019, we collected fecal samples within 65 areas mainly comprised of wildlife openings and fields across 489.62 km² of the NCWMA. We searched each area using a transect approximately 3,378 m long and 2

m wide. Areas smaller than the transect distance were searched in their entirety. We established a 1-week resting period between sampling events to allow time for fresh samples to reaccumulate. To minimize the potential for elk DNA and sample degradation, we waited approximately 1-day after heavy precipitation events before sampling designated collection areas (Brinkman et al. 2009). Based on sampling protocols in similar species, we recorded sample freshness by rating feces on a scale of 1–5 (1 being oldest and 5 being freshest) and collected samples with a rating 4 or 5 (Kirchhoff and Larsen 1998; Lupardus et al. 2011; Goode et al. 2014). We placed 10–15 fecal pellets in a bag and stored samples in a freezer (-20° C) until analysis.

Genetic Identification and Sex Determination

To collect elk DNA from fecal pellets, we removed samples from the freezer to thaw at room temperature for 15 minutes. Once partially thawed, we lightly rubbed the outer portions of 4 pellets with a flat toothpick and stored toothpicks in a labeled coin envelope. Similarly to Muller et al. (2018), we sent hair from captured individuals and 3 toothpicks per fecal sample (landscape samples) to Wildlife Genetics International (WGI; Nelson, British Columbia, Canada) for DNA analysis of 16 microsatellites and a sex marker for individual elk identification. Personnel at WGI analyzed samples using techniques outlined in Muller et al. (2018).

Blood Sample PSPB Analysis

To determine pregnancy status of captured females, we submitted 2 mL of whole blood to Herd Health Diagnostics (Pullman, Washington, USA) for PSPB testing using the BioPRYN Wild test (BioTracking, Moscow, Idaho, USA). The BioPRYN test

measures levels of PSPB in serum and is an accurate, reliable test for determining pregnancy status in animals ≥ 40 days post breeding (Noyes et al. 1997). Pregnancy status was determined using optical density values correlated to the amount of PSPB in a sample to differentiate pregnant (>0.210) and non-pregnant individuals (<0.135). We calculated the percent of pregnant females out of the total number of captured females for 2019 and 2020.

Fecal Sample EIA Analysis

We weighed out 0.2 g of ground fecal material to analyze fecal samples using enzyme immunoassay (EIA) methods to evaluate FPM concentration. Samples were dried for 24 hours at 50° C (Yamato Constant Temperature Oven DKN900, Orangeburg, New York, USA) and ground using a mortar and pestle. Progesterone metabolites were extracted from weighed samples using 200% absolute ethanol (Fisher Scientific, Hampton, New Hampshire, USA) and stored following the steroid solid extraction protocol recommended by Arbor Assays (Ann Arbor, Michigan, USA; Arbor Assays 2022). We added 1 mL ethanol for every 0.1 g fecal material, placed tubes on a rocking shaker for 30 min at room temperature, and centrifuged tubes for 16 minutes at 1792 g. We removed and stored the supernatant in 2 mL microcentrifuge tubes and assayed samples immediately or stored them up to 30 days at -20° C until analysis. Samples not assayed using both kits within 30 days of initial extraction were discarded and extracted again (Arbor Assays 2022).

We used 2 commercial EIA kits including Progesterone EIA (K025-H5; Arbor Assays) and Progesterone Metabolite (K068-H5; Arbor Assays) to quantify FPM

concentrations in extracted fecal samples. We followed kit protocols and read results at a wavelength of 450 nm using an iMark microplate absorbance reader (Bio-Rad, Hercules, California, USA). All samples and controls were run in duplicate. We conducted a test of parallelism for both kits by serially diluting fecal extract in assay buffer to produce 6 dilutions (1:50–1:150 range for Progesterone EIA kit, 1:20–1:640 range for Progesterone Metabolite kit). We compared the linear portions of the different diluted extracts of unknown samples with the progesterone standard binding curve for equality of slope (Ajó et al. 2022).

To assess any potential matrix interference within both kits, we calculated extraction efficiency of known amounts of progesterone in spiked fecal samples. For the spiked sample, we added 10 µl of 1,000 ng/mL progesterone standard for the Progesterone EIA kit and 15 µl of 1,000 ng/mL progesterone standard for the Progesterone Metabolite kit prior to extraction. Additionally, we prepared an unspiked sample with an amount of kit assay buffer equivalent to the amount of progesterone added to the spiked sample. Extraction efficiency was calculated by determining the difference between the spiked and unspiked sample concentrations and dividing by the concentration of the spiked sample.

We calculated inter-assay coefficient of variation (%CV) using the average concentration of 1 sample run in duplicate on all Progesterone EIA kit microplates ($n=19$) and Progesterone Metabolite kit microplates ($n=22$). We divided the standard deviation by the average FPM concentration of the sample and multiplied by 100. We calculated intra-assay %CV for each kit by averaging the %CV for each sample run on all

Progesterone EIA kit microplates ($n=19$) and Progesterone Metabolite kit microplates ($n=22$) and reported an overall mean for each kit. We excluded and reran samples if the duplicate measures had a $\%CV \geq 20$ and/or specific binding outside the range of 20-80%. Concentration results are expressed as ng of hormone per g of fecal powder (ng/g).

Statistical Analysis

We performed statistical analysis in RStudio (R Core Team 2021) and used a Welch Two Sample t -test to compare non-pregnant and pregnant FPM concentrations of captured females by assay kit. The 95% confidence interval (CI) from non-pregnant and pregnant captured females with both a blood and fecal sample were used to establish FPM concentration reference ranges. We used the determined reference range to categorize pregnancy status of female fecal samples collected non-invasively. We used a Fisher's exact test to evaluate whether pregnancy categorization of samples differed between the 2 kits. We used a linear mixed model for both the Progesterone EIA kit and the Progesterone Metabolite kit in the package lme4 (Bates et al. 2015). Models assessed how FPM concentrations of females with ≥ 2 samples collected changed over time. Individuals were assigned as a random effect to account for repeated samples collected across the sampling period and fixed effects included date and pregnancy status. To meet normality assumptions we log transformed FPM data. We obtained p -values for the fixed-effects using Analysis of Variance (ANOVA). We designated statistical significance at $P < 0.05$.

RESULTS

We collected 29 hair samples from captured elk and 357 fecal samples from the landscape for genetic analysis. We successfully identified individual genotype from all hair samples and 157 landscape fecal samples (fecal genotyping success rate of 44%). We captured 21 female elk (16 adults and 5 yearlings), collected a blood sample from 20 females, and collected a fecal sample from 17 females. We identified 128 female samples (62 unique individuals) from non-invasive fecal sampling. We used 16 of the 21 captured females (12 adults and 4 yearlings) with both a blood and fecal sample collected at the time of capture to establish FPM concentration reference ranges for non-pregnant and pregnant females. We included 7 male samples in the FPM analysis for comparison of FPM concentrations between the sexes.

Diluted sample progesterone metabolite concentrations for both kits were parallel to the standard curve (Ajó et al. 2022). The intra-assay %CV was 3.1% ($n=19$) for the Progesterone EIA kit and 5.1% ($n=22$) for the Progesterone Metabolite kit. The inter-assay %CV was 9.8% ($n=19$ microplates) for the Progesterone EIA kit and 16.3% ($n=22$ microplates) for the Progesterone Metabolite kit. Extraction efficiency was 93% for the Progesterone EIA kit and 85% for the Progesterone Metabolite kit.

Using PSPB from blood ($n=20$; 15 adults and 5 yearlings), we categorized 77% (8/11) adults in 2019 and 75% (3/4) adults in 2020 as pregnant. No yearling females ($n=5$) were pregnant. Using the Welch Two Sample t -test, we found a difference ($P=0.0009$) between non-pregnant ($\bar{x}=192.84 \pm 38.63$, 95% CI, 96.48–289.20) and pregnant ($\bar{x}=536.17 \pm 74.98$, 95% CI, 375.97–696.36) captured female FPM

concentrations with the Progesterone EIA kit (Fig. 2.1). For the Progesterone Metabolite kit we found a difference ($P=0.0008$) between non-pregnant ($\bar{x}=188.16 \pm 43.39$, 95% CI, 76.63–299.69) and pregnant ($\bar{x}=693.52 \pm 26.52$, 95% CI, 407.31–979.72) FPM concentrations (Fig. 2.1). There was no overlap identified between the 95% CI for non-pregnant and pregnant females, indicating a distinction between FPM concentrations of the 2 groups. Therefore, we used FPM concentration values of the 95% CI for non-pregnant and pregnant groups to categorize pregnancy status for unknown fecal samples. We classified fecal samples as undetermined when FPM concentrations were between the 95% CI ranges. Using the Progesterone EIA kit, we categorized 25 females as non-pregnant, 4 as undetermined, and 33 as pregnant (Table 2.1). Using the Progesterone Metabolite kit, we categorized 22 females as non-pregnant, 1 as undetermined, and 39 as pregnant (Table 2.1). All male sample ($n=7$) concentrations were similar to the FPM concentration range established for non-pregnant females.

Thirty-five females had ≥ 2 samples collected during the sampling period, with 28 having ≥ 2 samples collected on different days (Table 2.3., Table 2.4.). Twenty-five females with the Progesterone EIA kit and 29 females with the Progesterone Metabolite kit had all individual samples categorized with the same pregnancy status. Ten females with the Progesterone EIA kit and 6 females with the Progesterone Metabolite kit had at least 1 sample with a different pregnancy status categorization as compared to all other individual samples. In the linear mixed models for the Progesterone EIA kit and Progesterone Metabolite kit, the coefficient estimate for date was 0.02, indicating FPM concentrations of females categorized as non-pregnant and pregnant increased as the

sampling period progressed. Results from the ANOVA indicated a statistically significant, positive relationship between date and pregnancy status (Progesterone EIA kit: $P=0.00001$; Progesterone Metabolite kit: $P=0.000001$). With the Fisher's exact test, no difference in pregnancy categorization of samples was detected between the 2 kits ($P=0.34$).

DISCUSSION

We found pregnancy status of captured elk in 2019 and 2020 similar to the elk from Elk Island National Park used for reintroduction to Tennessee (Muller et al. 2004). Studies have shown pregnancy in elk populations vary widely. In eastern populations, percentages of pregnancy in elk were reported between 85–90% in Kentucky (Kentucky Department of Fish and Wildlife Resources (KDFWR) 2019) and 72–73% for elk ≥ 3 years old in the Great Smoky Mountains National Park (GSMNP; Murrow et al. 2009). Western elk populations have reported percentages between 50%–85% (Trainer 1971; Stoops et al. 1999; Creel et al. 2003; Proffitt et al. 2014).

Physical condition, nutrition, age of females and males, environment, and population density all affect reproduction and rate of pregnancy (Cook et al. 2013; Morano et al. 2013; Proffitt et al. 2014). None of the captured yearling elk at the NCWMA were pregnant, but yearling pregnancy is highly variable for different elk populations. In Kentucky, pregnancy in yearling elk were 10% in 2016 which increased to 71% in 2019 (KDFWR 2019). Percentages below 80% in adult elk 3.5–7.5 years of age may be indicative of reproductive issues within the population as adult females are expected to be highly fertile with the ability to successfully conceive and produce

offspring (Raedeke et al. 2002). Noyes et al. (1997) found higher percentages in elk populations with mature bulls as the predominant breeders. Male age structure and pregnancy status across age classes should be evaluated at the NCWMA as potential influencers of reproductive potential.

Through capture we determined pregnancy status in females using PSPB testing with a blood sample and provided a corresponding FPM concentration. We were able to use FPM concentrations from captured females with known age and pregnancy status to establish a reference range for categorizing females with an unknown pregnancy status from samples collected non-invasively across the landscape. Using genetic analysis for individual and sex identification, we were able to differentiate non-invasively collected samples, similarly to successful applications in reindeer (*Rangifer tarandus*; Morden et al. 2011). However, we could not differentiate between age classes and may have inadvertently included female calves and yearlings in our analysis. The potential inclusion of calves and yearlings may have inflated the number of females categorized as non-pregnant. Differentiating adult female age classes would improve pregnancy assessment. Using fecal pellet morphometrics for determining age class has been evaluated in reindeer (Morden et al. 2011) and captive mule deer (*Odocoileus hemionus*; Sánchez-Rojas et al. 2004) and may provide a cost effective alternative to observational methods, especially in heavily forested or mountainous areas. Further research evaluating the efficacy of utilizing fecal pellet morphometrics to differentiate age classes in elk is warranted. Our methodology, however, allowed us to successfully provide a minimum number of pregnant females (53.2% with the Progesterone EIA kit and 62.9% with the

Progesterone Metabolite kit) in the population with the use of non-invasive fecal sampling.

White et al. (1995) reported the most accurate measure for determining pregnancy status in elk was through free progesterone and pregnanediol-3-glucuronide in multiple fecal samples collected during late gestation. We collected samples during the mid to late gestational period of the NCWMA elk and saw significantly higher FPM concentrations in females categorized as pregnant versus non-pregnant. From females with repeat samples across the collection period, we identified an increase in FPM concentrations as sampling continued into late gestation.

While single samples can be used to determine pregnancy status during the late gestational period of elk, it does not reflect any physiological changes that may occur after sample collection. Monitoring individual FPM concentrations annually or across the gestational period on an individual basis may improve population level assessment by monitoring fluctuations or changes in individual pregnancy status. A majority of females with multiple fecal samples collected during our study had no change in pregnancy status and relatively similar FPM concentrations across individual samples. We were able to consistently categorize pregnancy status for 71.4% of females with the Progesterone EIA kit and 82.9% of females with the Progesterone Metabolite Kit using ≥ 2 samples collected from an individual.

Ten females with the Progesterone EIA kit and 6 females with the Progesterone Metabolite kit had at least 1 inconsistent sample with a different pregnancy status categorization as compared to all other collected samples. Potential issues during fecal

extraction or pipetting could explain the dissimilarities in these samples. The Progesterone Metabolite kit had higher %CV values as compared to the Progesterone EIA kit for females with inconsistencies across both kits and sample FPM concentrations, and therefore pregnancy status categorization. It is possible the Progesterone Metabolite kit may be more sensitive to human error occurring in the assay, possibly leading to higher %CV values in duplicates. Some samples were extracted twice between kit analyses due to unusable %CV values and/or percentage of binding during initial assays, or time between kit analyses was >30 days. Therefore, errors during fecal extractions or assaying as well as using different extractions for each kit potentially contributed to the dissimilarities in samples for females with inconsistent FPM concentrations or categorization by kit. Therefore, ensuring accuracy in sample processing and analysis and reducing %CV values in duplicates will help improve consistency of FPM concentration analyses and pregnancy status categorization of females with multiple samples collected across gestation (Palme et al. 2013).

Assay validation tests for both EIA kits provided reliable measurements of hormones present in the fecal extracts without being influenced by extraneous components within the extract (Morden et al. 2011). Cross reactivity with progesterone for both kits was 100%. However, the level of cross reactivity differed with various, similarly structured steroids. Cross reactivity for the Progesterone EIA kit was 172% for 3 β -hydroxy-progesterone, 188% for 3 α -hydroxy-progesterone, 147% for 11 α -hydroxy-progesterone, and <7% for pregnenolone, corticosterone, and androstenedione (Arbor Assays, Ann Arbor, Michigan, USA). Cross reactivity for the Progesterone Metabolite kit

did not exceed 62% for any closely associated steroids (Arbor Assays, Ann Arbor, Michigan, USA). Comparison of the 2 EIA kits indicated no significant difference in FPM concentrations between results of the Progesterone EIA kit and the Progesterone Metabolite kit. The Progesterone EIA kit showed higher precision compared to the Progesterone Metabolite kit. However, more females were categorized as undetermined as compared to the Progesterone Metabolite kit. Either kit was suitable for measuring FPM concentrations in elk feces to determine pregnancy status. Similarities between the kits resulted in no statistically significant difference between pregnancy status categorization and both are seemingly valid for measuring FPM concentrations in elk. However, the Progesterone EIA kit had lower intra-assay and inter-assay %CV between replicates, indicating a potentially better option for FPM evaluation.

Determining pregnancy status of elk at the NCWMA provides updated information on reproductive parameters that have not been evaluated since initial reintroduction. Non-invasive sampling as a method for pregnancy status determination can be used as an effective tool for population level assessment. Analyzing FPM concentrations can be used to obtain the same information as a blood sample with the benefit of non-invasive collection for obtaining samples across a wide landscape or gestational period (Schwarzenberger et al. 1997; Morden et al. 2011). More research to investigate methods for determining age class of female samples is warranted. Continued monitoring of pregnancy in conjunction with subsequent research on recruitment may provide valuable information needed to assess population growth potential.

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APPENDIX

Table 2.1. Pregnancy status categorization of female elk ($n=62$) at the North Cumberland Wildlife Management Area in Tennessee and male elk ($n=7$) fecal samples collected on the landscape in 2019 using Progesterone EIA and Progesterone Metabolite kits (Arbor Assays, Ann Arbor, Michigan). Pregnancy categorization was based on 95% confidence intervals of fecal progesterone metabolite concentrations from determined non-pregnant and pregnant captured female elk ($n=16$) in 2019 and 2020. Females with fecal progesterone metabolite concentrations between the ranges of non-pregnant and pregnant were categorized as undetermined.

Kit	Non-pregnant		Undetermined		Pregnant		Males	
	% Females (n)	ng/g	% Females (n)	ng/g	% Females (n)	ng/g	% Females (n)	ng/g
Progesterone EIA	36.5 (25)	(81.45 – 294.24)	1.6 (4)	(354.99 – 384.35)	53.2 (33)	(416.18 – 9140.84)	N/A (7)	(68.26 – 229.65)
Progesterone Metabolite	39.7 (22)	(56.68 – 282.43)	6.3 (1)	(331.57 – 372.68)	62.9 (39)	(379.86 – 4591.33)	N/A (7)	(105.83 – 297.88)

Table 2.2. Collection date, fecal progesterone metabolite concentration, and pregnancy status categorization using Arbor Assays Progesterone EIA kit of female elk ($n=35$) with ≥ 2 fecal samples collected during the 2019 non-invasive landscape collection at the North Cumberland Wildlife Management Area in Tennessee. Fecal progesterone metabolite concentrations are expressed as ng/g and samples were categorized as non-pregnant (NP), pregnant (P), or undetermined (U) based on the 95% confidence intervals established from determined non-pregnant and pregnant captured females.

Female		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Elk 1	ID	S29	S67			
	Date	15-Feb	26-Feb			
	ng/g	1330.61	66.87			
	Categorization	P	NP			
Elk 2	ID	S33	S273			
	Date	15-Feb	23-Feb			
	ng/g	138.89	144.56			
	Categorization	NP	NP			
Elk 3	ID	S31	S105			
	Date	15-Feb	5-Mar			
	ng/g	487.63	500.75			
	Categorization	P	P			
Elk 4	ID	S110	S349			
	Date	5-Mar	24-Apr			
	ng/g	685.4505	1115.6665			
	Categorization	P	P			
Elk 5	ID	S27	S106			
	Date	15-Feb	5-Mar			
	ng/g	89.8	204.84			
	Categorization	NP	NP			
Elk 6	ID	S32	S90			
	Date	15-Feb	27-Feb			
	ng/g	85.53	83.24			
	Categorization	NP	NP			
Elk 7	ID	S36	S222			
	Date	15-Feb	19-Mar			
	ng/g	384.66	585.38			
	Categorization	P	P			

Table 2.2 continued

Female		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Elk 8	ID	S40	S75			
	Date	15-Feb	27-Feb			
	ng/g	282.43	379.86			
	Categorization	NP	P			
Elk 9	ID	S109	S145			
	Date	5-Mar	7-Mar			
	ng/g	584.65	358.06			
	Categorization	P	U			
Elk 10	ID	S125	S343			
	Date	5-Mar	18-Apr			
	ng/g	107.86	502.46			
	Categorization	NP	P			
Elk 11	ID	S56	S126			
	Date	26-Feb	5-Mar			
	ng/g	213.97	364.38			
	Categorization	NP	U			
Elk 12	ID	S175	S211			
	Date	12-Mar	14-Mar			
	ng/g	669.89	607.61			
	Categorization	P	P			
Elk 13	ID	S205	S229			
	Date	14-Mar	19-Mar			
	ng/g	875.48	1295.39			
	Categorization	P	P			
Elk 14	ID	S215	S320			
	Date	19-Mar	11-Apr			
	ng/g	423.65	1255.29			
	Categorization	P	P			
Elk 15	ID	S66	S312			
	Date	26-Feb	3-Apr			
	ng/g	262.58	705.31			
	Categorization	NP	P			
Elk 16	ID	S101	Elk#4			
	Date	5-Mar	26-Feb			
	ng/g	409.72	489.29			
	Categorization	P	P			

Table 2.2 continued

Female		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Elk 17	ID	S276	Elk#6			
	Date	28-Feb	23-Mar			
	ng/g	129.63	285.28			
	Categorization	NP	NP			
Elk 18	ID	S221	Elk#7			
	Date	28-Feb	19-Mar			
	ng/g	652.35	630.85			
	Categorization	P	P			
Elk 19	ID	S339	S341			
	Date	18-Apr	18-Apr			
	ng/g	384.19	1742.22			
	Categorization	P	P			
Elk 20	ID	S100	S103			
	Date	5-Mar	5-Mar			
	ng/g	435.54	372.68			
	Categorization	P	U			
Elk 21	ID	S324	S325			
	Date	18-Apr	18-Apr			
	ng/g	140.5	248.81			
	Categorization	NP	NP			
Elk 22	ID	S79	S81			
	Date	27-Feb	27-Feb			
	ng/g	258.99	244.12			
	Categorization	NP	NP			
Elk 23	ID	S281	S285			
	Date	23-Mar	23-Mar			
	ng/g	482.28	564.53			
	Categorization	P	P			
Elk 24	ID	S323	S334			
	Date	18-Apr	18-Apr			
	ng/g	2609.97	1550.54			
	Categorization	P	P			
Elk 25	ID	S239	S244			
	Date	19-Mar	19-Mar			
	ng/g	664.56	673.62			
	Categorization	P	P			

Table 2.2 continued

Female		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Elk 26	ID	S26	S167	S209		
	Date	15-Feb	7-Mar	14-Mar		
	ng/g	334.8	557.74	333.35		
	Categorization	U	P	U		
Elk 27	ID	S37	S224	S227		
	Date	15-Feb	19-Mar	19-Mar		
	ng/g	361.55	643.34	617.75		
	Categorization	U	P	P		
Elk 28	ID	S63	S338	S347		
	Date	26-Feb	18-Apr	18-Apr		
	ng/g	372.66	1947.11	4026.01		
	Categorization	U	P	P		
Elk 29	ID	S77	S80	S280		
	Date	27-Feb	27-Feb	23-Mar		
	ng/g	64.78	85.7	54.96		
	Categorization	NP	NP	NP		
Elk 30	ID	S42	S78	S82		
	Date	15-Feb	27-Feb	27-Feb		
	ng/g	62.35	115.64	68.75		
	Categorization	NP	NP	NP		
Elk 31	ID	S328	S332	S356		
	Date	18-Apr	18-Apr	25-Apr		
	ng/g	2431.89	3344.51	2399.3		
	Categorization	P	P	P		
Elk 32	ID	S48	S49	S50	S213	
	Date	26-Feb	26-Feb	26-Feb	19-Mar	
	ng/g	67.14	65.35	83.51	134.23	
	Categorization	NP	NP	NP	NP	
Elk 33	ID	S124	S306	S311	S344	
	Date	5-Mar	3-Apr	3-Apr	18-Apr	
	ng/g	650.51	1091.9	1851.58	1550.54	
	Categorization	P	P	P	P	
Elk 34	ID	S28	S228	S232	S233	S240
	Date	15-Feb	19-Mar	19-Mar	19-Mar	19-Mar
	ng/g	395.84	516.04	671.89	575.58	780.59
	Categorization	P	P	P	P	P

Table 2.2 continued

Female		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Elk 35	ID	S303	S331	S333	S354	S357
	Date	3-Apr	18-Apr	18-Apr	25-Apr	25-Apr
	ng/g	1414.27	1983.64	2170.07	1546.34	1608.31
	Categorization	P	P	P	P	P

Table 2.3. Collection date, fecal progesterone metabolite concentration, and pregnancy status categorization using Arbor Assays Progesterone Metabolite kit of female elk ($n=35$) with ≥ 2 fecal samples collected during the 2019 non-invasive landscape collection at the North Cumberland Wildlife Management Area in Tennessee. Fecal progesterone metabolite concentrations are expressed as ng/g and samples were categorized as non-pregnant (NP), pregnant (P), or undetermined (U) based on the 95% confidence intervals established from determined non-pregnant and pregnant captured females.

Female		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Elk 1	ID	S29	S67			
	Date	15-Feb	26-Feb			
	ng/g	175.03	200.16			
	Categorization	NP	NP			
Elk 2	ID	S33	S273			
	Date	15-Feb	23-Feb			
	ng/g	438.66	271.32			
	Categorization	P	NP			
Elk 3	ID	S31	S105			
	Date	15-Feb	5-Mar			
	ng/g	1020.35	659.24			
	Categorization	P	P			
Elk 4	ID	S110	S349			
	Date	5-Mar	24-Apr			
	ng/g	494.34	1485.49			
	Categorization	P	P			
Elk 5	ID	S27	S106			
	Date	15-Feb	5-Mar			
	ng/g	141.22	142.85			
	Categorization	NP	NP			
Elk 6	ID	S32	S90			
	Date	15-Feb	27-Feb			
	ng/g	181.98	158			
	Categorization	NP	NP			
Elk 7	ID	S36	S222			
	Date	15-Feb	19-Mar			
	ng/g	997.41	656.78			
	Categorization	P	P			

Table 2.3 continued

Female		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Elk 8	ID	S40	S75			
	Date	15-Feb	27-Feb			
	ng/g	361.38	532.99			
	Categorization	U	P			
Elk 9	ID	S109	S145			
	Date	5-Mar	7-Mar			
	ng/g	611.44	434.47			
	Categorization	P	U			
Elk 10	ID	S125	S343			
	Date	5-Mar	18-Apr			
	ng/g	147.33	441.15			
	Categorization	NP	P			
Elk 11	ID	S56	S126			
	Date	26-Feb	5-Mar			
	ng/g	555.61	321.09			
	Categorization	P	U			
Elk 12	ID	S175	S211			
	Date	12-Mar	14-Mar			
	ng/g	1602.75	970.62			
	Categorization	P	P			
Elk 13	ID	S205	S229			
	Date	14-Mar	19-Mar			
	ng/g	1879.7	5225.3			
	Categorization	P	P			
Elk 14	ID	S215	S320			
	Date	19-Mar	11-Apr			
	ng/g	513.38	4077.63			
	Categorization	P	P			
Elk 15	ID	S66	S312			
	Date	26-Feb	3-Apr			
	ng/g	1000.77	1083.16			
	Categorization	P	P			
Elk 16	ID	S101	Elk#4			
	Date	5-Mar	26-Feb			
	ng/g	755.68	672.03			
	Categorization	P	P			

Table 2.3 continued

Female		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Elk 17	ID	S276	Elk#6			
	Date	28-Feb	23-Mar			
	ng/g	252.17	240.6			
	Categorization	NP	NP			
Elk 18	ID	S221	Elk#7			
	Date	28-Feb	19-Mar			
	ng/g	1015.92	1147.46			
	Categorization	P	P			
Elk 19	ID	S339	S341			
	Date	18-Apr	18-Apr			
	ng/g	698.6	2190.92			
	Categorization	P	P			
Elk 20	ID	S100	S103			
	Date	5-Mar	5-Mar			
	ng/g	713.35	548.97			
	Categorization	P	P			
Elk 21	ID	S324	S325			
	Date	18-Apr	18-Apr			
	ng/g	326.21	301.37			
	Categorization	U	U			
Elk 22	ID	S79	S81			
	Date	27-Feb	27-Feb			
	ng/g	576.28	355.96			
	Categorization	P	U			
Elk 23	ID	S281	S285			
	Date	23-Mar	23-Mar			
	ng/g	892.98	870.43			
	Categorization	P	P			
Elk 24	ID	S323	S334			
	Date	18-Apr	18-Apr			
	ng/g	7692.21	9140.84			
	Categorization	P	P			
Elk 25	ID	S239	S244			
	Date	19-Mar	19-Mar			
	ng/g	1052	449.46			
	Categorization	P	P			

Table 2.3 continued

Female		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Elk 26	ID	S26	S167	S209		
	Date	15-Feb	7-Mar	14-Mar		
	ng/g	636.19	1012.73	384.21		
	Categorization	P	P	U		
Elk 27	ID	S37	S224	S227		
	Date	15-Feb	19-Mar	19-Mar		
	ng/g	710.38	797.64	866.94		
	Categorization	P	P	P		
Elk 28	ID	S63	S338	S347		
	Date	26-Feb	18-Apr	18-Apr		
	ng/g	548.96	4223.35	7986.65		
	Categorization	P	P	P		
Elk 29	ID	S77	S80	S280		
	Date	27-Feb	27-Feb	23-Mar		
	ng/g	82.53	42.27	119.55		
	Categorization	NP	NP	NP		
Elk 30	ID	S42	S78	S82		
	Date	15-Feb	27-Feb	27-Feb		
	ng/g	134.29	131.84	69.5		
	Categorization	NP	NP	NP		
Elk 31	ID	S328	S332	S356		
	Date	18-Apr	18-Apr	25-Apr		
	ng/g	2818.31	5093.84	2084.53		
	Categorization	P	P	P		
Elk 32	ID	S48	S49	S50	S213	
	Date	26-Feb	26-Feb	26-Feb	19-Mar	
	ng/g	139.56	79.37	237.38	131.25	
	Categorization	NP	NP	NP	NP	
Elk 33	ID	S124	S306	S311	S344	
	Date	5-Mar	3-Apr	3-Apr	18-Apr	
	ng/g	642.44	1132.91	1808.82	2610.95	
	Categorization	P	P	P	P	
Elk 34	ID	S28	S228	S232	S233	S240
	Date	15-Feb	19-Mar	19-Mar	19-Mar	19-Mar
	ng/g	565.93	1124.28	598.12	967.88	663.06
	Categorization	P	P	P	P	P

Table 2.3 continued

Female		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Elk 35	ID	S303	S331	S333	S354	S357
	Date	3-Apr	18-Apr	18-Apr	25-Apr	25-Apr
	ng/g	2430.15	810.2	3062.23	2796.99	2108.95
	Categorization	P	P	P	P	P

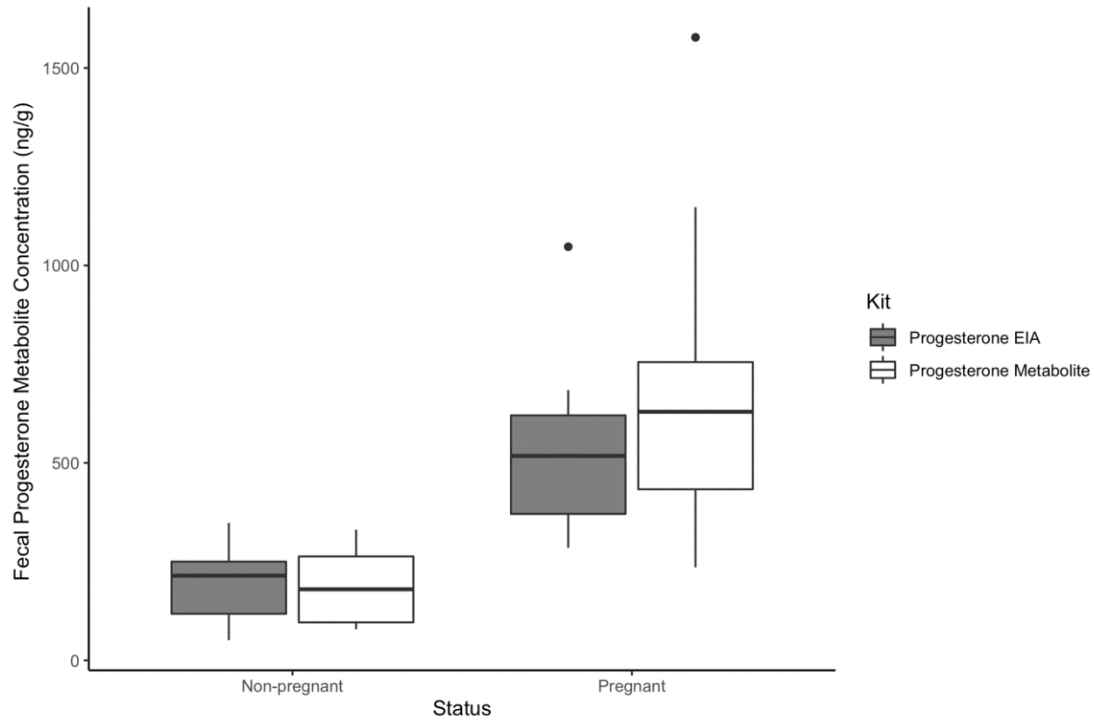


Figure 2.1. Boxplot of the mean fecal progesterone metabolite concentrations of non-pregnant and pregnant female elk ($n=16$) captured at the North Cumberland Wildlife Management Area in Tennessee (2019–2020) using the Progesterone EIA kit and Progesterone Metabolite kit (Arbor Assays, Ann Arbor, Michigan). Pregnancy was determined based on Pregnancy-specific protein B data. Grey boxes represent the Progesterone EIA kit and white boxes represent the Progesterone Metabolite kit.

CONCLUSION

The goals of this research were to provide more in depth knowledge of population parameters related to genetics and reproduction of North Cumberland Wildlife Management Area (NCWMA) elk in Tennessee. Genetic variability was evaluated 11+ years after reintroduction and indicated the persistence of the 2 original Elk Island National Park (EINP) genetic lineages used for Tennessee reintroductions. Pregnancy rates were determined during initial elk releases, but not measured after elk were established. This research evaluated genetic variability, relatedness, spatial distribution and segregation of populations, pregnancy rates of captured adult females, and the efficacy of non-invasive fecal sampling for detecting pregnancy on a population scale.

We captured 29 elk and collected 357 fecal samples non-invasively on the landscape for genetic evaluation. We used DNA analysis of 16 microsatellites and a sex marker to genetically identify 101 unique individuals (26 males and 75 females) from hair and fecal samples. We used program STRUCTURE to evaluate current population structure and assigned elk to EINP North ($n=22$), EINP South ($n=62$), or admixture ($n=17$) groups. We determined an increase in both EINP South and admixture and possible indications of a decline in the EINP North genetic group. We used program CERVUS to evaluate genetic diversity and found minimal changes since previous evaluation. We used the R packages related and Hierfstat to evaluate genetic relatedness. We found low genetic differentiation (indicating gene flow was occurring) in the populations. The EINP North elk had higher relatedness compared to elk in EINP South and admixture groups.

We used GPS locations recorded during fecal collection and from collared elk to evaluate spatial distribution and estimate home range size of the genetic groups. We determined the EINP genetic groups are largely segregated spatially, with high site fidelity and small annual and breeding season home ranges. Female pairs in close proximity were more closely related. Social dynamics, strong site fidelity, and positive assortative mating were possible mechanisms for genetic structuring of the NCWMA population.

Pregnancy rates are needed for understanding population growth potential. We captured 21 female elk (16 adults and 5 yearlings) and collected hair, feces, and blood samples from individuals. Pregnancy of captured adult females was 72.7% (8/11) in 2019 and 75% (3/4) in 2020 using pregnancy-specific protein B (PSPB) testing from blood samples. From 16 of the 21 captured females (12 adults and 4 yearlings) with both a blood (PSPB concentrations) and fecal sample collected concurrently at capture, we established a fecal progesterone metabolite (FPM) concentration reference range for non-pregnant and pregnant females using Arbor Assays Progesterone EIA kit and Progesterone Metabolite kit. Progesterone EIA kit FPM concentrations were $\bar{x}=192.84 \pm 38.63$ ng/g (95% CI, 96.48–289.20) for non-pregnant and $\bar{x}=536.17 \pm 74.98$ ng/g (95% CI, 375.97–696.36) for pregnant females. Progesterone Metabolite kit FPM concentrations were $\bar{x}=188.16 \pm 43.39$ ng/g (95% CI, 76.63–299.69) for non-pregnant and $\bar{x}=693.52 \pm 126.52$ ng/g (95% CI, 407.31–979.72) for pregnant females. We conducted population level assessment of FPM concentrations from fecal samples collected on the landscape to determine pregnancy from individual females (identified from the fecal DNA). We identified 128 female samples and 62 unique females. We found 35 of the 62 females had ≥ 2 samples collected across the sampling period.

Although we were unable to determine age class from non-invasively collected fecal samples, we successfully categorized female samples with an unknown pregnancy status as non-pregnant, undetermined, or pregnant using the FPM reference range established from captured females with the Progesterone EIA kit (53.2%) and Progesterone Metabolite kit (62.9%). Using individuals with ≥ 2 samples, we consistently categorized pregnancy status for 71.4% of females with the Progesterone EIA kit and 82.9% of females with the Progesterone Metabolite Kit. Our research determined non-invasive fecal sampling can provide a minimum number of pregnant females on the landscape. Continued monitoring of pregnancy with calf survival and recruitment would provide valuable information needed to assess NCWMA population growth potential.

VITA

Eryn M. Watson received her bachelor's degree in Animal Science and her master's degree in Forest Resources with an emphasis on Wildlife Management and Ecology at the University of Georgia. She conducted research on the reintroduced elk population in Tennessee and focused on population parameters related to genetics and reproduction for her PhD dissertation.