PROJECT TITLE: Preparation of manuscripts on wolf ecology in Interior Alaska

PRINCIPAL INVESTIGATOR: Mark McNay

COOPERATORS: None

FEDERAL AID GRANT PROGRAM: Wildlife Restoration

GRANT AND SEGMENT NR: W-33-2 and W-33-3

PROJECT NR: 14.22

WORK LOCATION: Fairbanks

STATE: Alaska

PERIOD: 1 JULY 2004–30 JUNE 2005

I. PROBLEM OR NEED THAT PROMPTED THIS RESEARCH

Between 1995 and 2001 the Alaska Department of Fish and Game monitored wolf population dynamics in Game Management Unit 20A. Public hunting and trapping in some years regulated wolf population growth, but sex and age composition of the harvest appeared to be important in determining the regulatory effect of the harvest (McNay 2002). High exploitation rates alone did not ensure population regulation.

High pregnancy rates were diagnosed in both live captured female wolves, and postmortem samples. Ultrasound diagnoses of live captured wolves revealed that more than one female was pregnant in several of the sampled wolf packs. Those findings, in part, explained the population's 21% increase in a single year when more than 30% of the population was harvested by public hunting and trapping. However, it remained unclear if inbreeding, possibly as a consequence of social disruption, contributed to multiple pregnancies and increased population productivity.

In winters of 1998 and 2000 we developed and tested a periodic sampling design to estimate predation rates by wolves on moose and caribou during winter (McNay and Ver Hoef 2003). The sampling method avoids biases associated with seasonal sampling. We found seasonal variation in overall kill rates and variation in per capita kill rates related to pack size. Per capita kill rate was inversely related to pack size. Therefore, increased harvest could increase per capita kill rates by wolves, and overall predation rates may not decline despite reductions in wolf population size.

Findings from both of these studies had not been published in the peer-reviewed literature but represented important concepts in the management of wolf-prey systems. Therefore, the writing project was proposed to allow the principal investigator time to prepare 2 manuscripts for publication and a separate report on the results of paternity testing.

II. REVIEW OF PRIOR RESEARCH AND STUDIES IN PROGRESS ON THE PROBLEM OR NEED

Pup production and survival are the most significant factors contributing to wolf (*Canis lupus*) population growth (Fuller et al. 2003), but precisely measuring those attributes in free-ranging wolf populations is difficult. Observations of breeding are infrequent and unreliable as a measure of conception date because conception may occur several days after breeding (Concannon et al. 1983), and female wolves may copulate numerous times during their 1–2 week receptive period (Packard 2003). Blood tests, commonly used to detect pregnancy in ungulates (Haigh et al. 1982; Plotka et al. 1977; Weber et al. 1982), are inaccurate in wolves because serum progesterone levels are similar in pregnant and nonpregnant females (Kreeger 2003). Consequently, pregnancy rates and parturient litter size are commonly estimated from postmortem examinations (Fuller et al. 2003; Rausch 1967). However, we needed estimates of pregnancy and pup survival among live females of known social status to explain effects of exploitation on pack productivity.

High pregnancy rates and the potential for multiple litters in wolf packs have been found in heavily hunted and trapped wolf populations (Rausch 1967; Ballard et al. 1987). Woolpy (1968) suggested exploitation caused changes in wolf social structure and a breakdown of socially induced breeding restrictions, but Mech et al. (1998) reported multiple litters in an unexploited wolf population. Haber (1996) suggested inbreeding was a common characteristic of unexploited populations. If so, then multiple litters in unexploited populations would commonly result from alpha males breeding their subordinate female daughters.

III. APPROACHES USED AND FINDINGS RELATED TO THE OBJECTIVES AND TO PROBLEM OR NEED

OBJECTIVE 1: Prepare a scientific manuscript on reproductive characteristics of an exploited wolf population.

A manuscript was written and submitted to the Journal of Mammalogy documenting the accuracy, efficacy, and safety of ultrasound in estimating pregnancy and litter size in live capture wolves. The manuscript also presented data on fetal growth rates and on the determination of litter size from placental scars in postmortem samples. The manuscript was accepted and scheduled for publication in February 2006, the accepted abstract is presented in the appendix of this report.

OBJECTIVE 2: Prepare a scientific manuscript on the use of periodic sampling to estimate predation rates by wolves on moose and caribou during winter.

No progress was made on this objective.

OBJECTIVE 3: Identify if single or multiple paternity existed among multiple litter wolf packs that were identified during federal aid study 14.17

Samples from 123 live-captured wolves were submitted for genetic analysis to Wildlife Genetics International (WGI), a commercial diagnostic laboratory. DNA extraction and initial parentage analysis was completed by WGI. Sixty-six offspring of 10 male and 15 female parents in 13 packs were genetically identified based on mismatch distributions of 22 loci genotypes.

We found no evidence of multiple paternity among our sampled packs. The primary male sired all sampled pups in both single and multiple litter packs identified by genetic analysis. All reproductive females identified in both single and multiple litter packs were not daughters of the current primary male; however, all productive secondary females were daughters of the current primary female.

A report on the genetic analysis of parentage in wolves was prepared and is included in the appendix of this report.

IV. MANAGEMENT IMPLICATIONS

The findings in the manuscript described for Objective 1 demonstrate that female wolves live-captured by darting from helicopters in the second trimester of pregnancy can be accurately and safely diagnosed for pregnancy using ultrasound. Use of placental scars from postmortem examinations of wolves can also be used to assess pregnancy, but variability in scar shading can lead to erroneous estimates of litter size. The manuscript presents analysis of differences in scar shading related to litter size determined by ultrasound and provides guidance to biologists attempting to estimate litter size from postmortem samples. Regressions for fetal growth are also presented in the manuscript and can be used to estimate date of breeding and expected date of parturition based on samples from either postmortem examinations, or from live ultrasound diagnoses.

The findings from the genetic analysis of parentage discount the hypothesis that inbreeding occurs within wolf packs. The findings also suggest that changes in social structure resulting from either natural or human-caused mortality can facilitate production of multiple litters within wolf packs, thereby increasing reproductive rates that can compensate for mortality.

V.   SUMMARY OF WORK COMPLETED ON JOBS IDENTIFIED IN ANNUAL PLAN FOR LAST SEGMENT PERIOD ONLY

JOB 1: Prepare the following 2 manuscripts for publication in scientific journals:

1) Reproductive characteristics of an exploited wolf population

A manuscript entitled, "Diagnosing Pregnancy, Inutero Litter Size, and Fetal Growth with Ultrasound in Wild, Free-ranging Wolves" was prepared and submitted to the Journal of Mammalogy. The manuscript was peer-reviewed; revisions were made and submitted. The manuscript is scheduled for publication in February 2006.

2) Estimating predation rates by wolves during winter with periodic sampling

At beginning of the reporting period the principal investigator was reassigned to serve as acting research coordinator for Interior Alaska. As a result, work on the predation rate manuscript was postponed. Expenditure of funds on study 14.22 was reduced, and salary money for the principal investigator came from other sources.

No further progress was made on the predation rate manuscript. The final performance report (McNay and Ver Hoef 2003) summarized study results. The wolf predation manuscript was included in a new project (14.23), due 1 September 2006.

JOB 2: Submit whole blood samples to a commercial diagnostic laboratory for genetic analysis of paternity and report results of paternity analysis on 3–4 packs identified as producing multiple litters.

The principal investigator wrote a report summarizing results of genotype and parentage analysis from 123 wolves.

VI. ADDITIONAL FEDERAL AID-FUNDED WORK NOT DESCRIBED ABOVE THAT WAS ACCOMPLISHED ON THIS PROJECT DURING THE LAST SEGMENT PERIOD, IF NOT REPORTED PREVIOUSLY

No additional work was completed.

VII.   PUBLICATIONS

One manuscript and one report resulted from this study. An abstract of the manuscript on reproductive characteristics follows here. The complete text of the report on parentage analysis is given in the appendix.

DIAGNOSING PREGNANCY, IN UTERO LITTER SIZE, AND FETAL GROWTH WITH ULTRASOUND IN WILD, FREE-RANGING WOLVES

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We document the accuracy, efficacy, and safety of ultrasound in estimating reproductive characteristics of gray wolves (*Canis lupus*) in Central Alaska. We live-captured 68 adult female wolves during the 2nd trimester of pregnancy and examined each with portable ultrasound equipment to diagnose pregnancy and litter size. Seventy-two percent were pregnant. In utero litter sizes ranged from 1 to 9 pups. We compared ultrasound diagnoses with postmortem embryo or placental scar counts in 14 females that died within 10 months after being examined by ultrasound; all ultrasound and postmortem examinations agreed in the diagnoses of pregnancy. Among 12 pregnant females, 6 agreed exactly in fetal count, 11 were within 1 fetus and all were within 2 fetuses. The shading of placental scars varied between individual wolves, but there was a general decline in placental scar color density between mid-September and mid-February. We describe a protocol for estimating litter size from placental scars. Radiocollared females were monitored from the air to estimate denning rates. Distance from the den declined as parturition approached, but few females localized near dens before parturition. Among 46 pregnant females diagnosed by ultrasound, 80.4% entered and remained at dens, 15.2% failed to enter dens and 4.4% denned but abandoned the den within 1 week. None of the females diagnosed as nonpregnant entered dens. We present models of fetal growth from ultrasound measurements of embryonic vesicle diameters (EVD) or crown rump length (CRL) of in utero fetuses. CRL was a better predictor of gestational age ($r^2 = 0.92$) than was EVD ($r^2 = 0.79$). We found no evidence that capture of females during the 2nd trimester of pregnancy affected denning or productivity.

(Current citation: McNay, M.E., T.R. Stephenson, and B.W. Dale. 2006. Diagnosing pregnancy, in utero litter size, and fetal growth with ultrasound in wild, free ranging wolves. Journal of Mammalogy 87(1): in press.)

VIII. RESEARCH EVALUATION AND RECOMMENDATIONS

IX. PROJECT COSTS FROM LAST SEGMENT PERIOD ONLY

Stewardship Investment items purchased: *list any equipment or other items purchased for which the cost of the individual item was $5,000 or more (include cost)*
None

FEDERAL AID SHARE = $28,875 STATE SHARE = $ 9,625 TOTAL =$38,500

X. APPENDIX

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PRELIMINARY RESULTS OF PARENTAGE ANALYSIS USING MICROSATELLITE MARKERS FROM AN EXPLOITED WOLF POPULATION IN CENTRAL ALASKA

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Abstract: Parentage and familial relationships within a highly exploited wolf population in central Alaska were examined to investigate how social disruption affects wolf reproductive performance. Skin tissue or blood samples were collected from 123 live captured wolves. DNA extraction, genotyping, and parentage analyses were performed by Wildlife Genetics International, Nelson B.C. Twenty-two locus genotypes were obtained from all samples. Parentage analysis was performed for both parents by plotting 2-parent mismatch distributions for all potential offspring. Parent offspring relationships were identified for 66 offspring from 10 male and 15 female parents. Multiple litters were identified genetically in 2 packs, in both cases a single male, the primary male, sired the multiple litters. In one pack 3 different females produced surviving offspring. In all cases, females that produced surviving offspring in both single litter and multiple litter packs were not daughters of the primary male. Production of multiple litters within our study area resulted when a primary male was replaced and secondary females sired by the previous primary male were retained within the pack.

Introduction

High pregnancy rates and multiple litters in single packs have been found in heavily hunted and trapped wolf populations (Rausch 1967; Ballard et al 1987). Woolpy (1968) suggested exploitation caused a breakdown of socially induced breeding restrictions, allowing pregnancy in several females within a single pack. However, multiple litters also occur in unexploited wolf populations (Mech et al 1998) and Haber (1996) suggested inbreeding was a common characteristic of unexploited populations. If so, then multiple litters could result from primary males breeding their subordinate female daughters. Alternatively, multiple litters could result from matings between subordinate females and males from different packs. Meir et al. (1995) found genetic variation

within the packs of Denali National Park that indicated genetic exchange between packs. That could result either from adoption of non pack members or by inter pack breeding. However, to date no one has identified the genetic relationships among multiple breeding females, their offspring and potential fathers of those offspring within multiple litter wolf packs.

We studied familial relationships among wolves in a highly exploited wolf population in central Alaska between 1995 and 2001. This report provides preliminary results of genetic based parentage analysis among wolves that were live captured during our study.

Study Area

Our study area (11,500 km^2, 64° 10´ N 147° 45´ W) within Alaska's Game Management Unit 20A (GMU 20A) was the site of previous studies on moose, caribou, and wolves. (Gasaway et. al 1983, Boertje et al. 1996, Valkenburg, et al. 2004.). Elevations range from 300m to 4000 m sloping upward north to south from poorly drained "flats" of boreal spruce/birch forest (*Picea spp., Betula spp.*), through a foothill zone of alpine shrubs(*Salix* spp*. Alnus* spp*, Betula* spp.*Populus* spp) and tundra sedges (Carex spp., Eriophorum spp.) to the crest of the Alaska Range. The terrain above 2000m is mostly rock covered and supports little vegetation with areas of permanent snow or glacial ice. The study area is roadless except for seasonal mining trails and trails to homestead sites along the western boundary. Hunting for wolves in GMU 20A was allowed from 10 August - 30 April and trapping was allowed from 1 November to 30 April. Denali National Park lies adjacent to the study area and wolves are protected within the Park.

Methods

From March 1995 through March 2000 we live-captured wolves by darting the animals from helicopters with 3cc Palmer Cap-Chur[®] (Palmer Cap-Chur Equipment, Douglasville, Georgia) darts loaded with 500–560 mg of Telazol[®](tiletamine HCl and zolazepam HCl, Fort Dodge Lab, Fort Dodge, Iowa), and propelled by low velocity (brown) charges. We attached numbered ear tags to all live captured wolves and fitted mortality-sensing radio collars to most. (Telonics, Inc, Mesa Arizona USA). From each captured wolf we recorded weight, gender, and various body measurements. We collected whole blood and punched an approximately 3mm diameter disk of skin, cartilage and hair from the ear to apply ear tags. Ear punch samples were air dried in paper envelopes, frozen and stored in plastic cryotubes. Samples were shipped to Wildlife Genetics International (WGI, Nelson, British Columbia) for DNA extraction, genotyping and preliminary parentage analysis. WGI developed 22 microsatellite markers for this project using the following criteria for acceptable markers:

1) Marker had to be mapped to chromosome, and no chromosome could contribute more than one marker to insure markers were not linked.

2) The microsatellite repeat had to contain at least 17 uninterrupted tandem repeats to insure variability

3) Repetitive sequences on either side of the core repeat sequence had to be minimal to reduce the chance for compound variation

4) Total length of amplified sequences had to be < 250 base pairs because shorter lengths of DNA are more likely to amplify from poor quality samples.

5) Primer sequences had to produce strong, legible results.

WGI used the exclusion method for parentage analysis, in which hypothesized parent-offspring sets that did not have matching alleles at all examined loci were excluded (Jones and Arden 2003). Our data set was suitable for that analysis because the sample came from an intensively studied population in which putative relationships were already identified. I used cementum or known ages of sample wolves to eliminate nonsensical parent offspring relationships that remained after the exclusion analysis.

I use the terms primary and secondary rather than the more traditional terms "alpha" and "subordinate" to differentiate social status among reproductive aged females (i.e. ≥22 months) within a single pack. Primary females exhibited high pack fidelity, were associated with the primary male more often than other pack members during winter and early spring, and were the oldest females within a pack. Primary females did not disperse or exhibit extraterritorial movements alone. Secondary females were younger than the primary, often exhibited predisperal movements outside of their territory, and most eventually dispersed from multiple female packs.

Pups (≤11 months of age) were identified by incomplete eruption of canine teeth and by the prominent swelling at the distal end of the radius that indicated incomplete ossification of the metaphysis. I identified yearling females (12–23 months of age) from known ages if they had been initially captured as pups, by tooth cementum age from the 1st upper premolar (Ballard et al. 1995) if a postmortem sample was available, or by using a combination of nipple size (Mech et al. 1993) and tooth wear similar to that described by Gipson et al. (2000). Live captured animals lacking pup characteristics were considered yearlings if they had slight or no wear on incisors and a combined width + length nipple measurement of less than 8 mm. The 8-mm value was assigned because it was below the 90% confidence interval (8.3–10.6) of the mean nipple size of cementum aged and known aged 29- to 36-month-old wolves ($n = 9$) in the sample.

Results

WGI scored 22 locus genotypes for 123 wolves live captured in GMU 20A between 1995 and 1999. Heterozygosity in the 22 loci genotypes averaged 0.76 with an average of 5.6 alleles per locus (Table 1). Parentage was identified for 66 offspring based on complete 22 loci matches with candidate mother-father pairs (Table 2)

Ten primary males and 10 primary females were identified in 11 packs based on genotypes with supporting evidence from behavior of wolves observed during radio tracking, tenure within the pack, and relative ages . Five productive secondary females were identified in 3 of those packs. Multiple

litters in a single pack in the same year were confirmed in 2 packs (Pack #7 and Pack #8), in each case the multiple litters were sired by a single male, the primary male.

None of the 5 secondary females identified as mothers in multiple litter packs were daughters of the primary male, therefore we found no evidence of inbreeding. However, in all 5 cases the secondary females were the daughters of the primary female. We identified one secondary female (#190) that was not the daughter of the primary female (139). That secondary was confirmed pregnant by ultrasound in both 1996 and 1997 (McNay et al 2006), but our only genetic sample from a pup (331)in that pack during those years came from an offspring of the primary female (139). Therefore we could not document production of pups by unrelated females within the same pack during the same year.

Parentage analysis indicated female 462 produced surviving pups in two different packs. First as a secondary female in the Jumbo pack (pack #7) she produced pup 187 in 1995. During the same year the Jumbo pack primary female (199) also produced pups (185, 186). Female 462 then dispersed and became the primary female in the Boulder Creek pack (pack # 46), those pups were sired by male 150. Male 150 had sired pups in the adjacent Mystic Creek pack (pack #5) in 1995, but after the primary female was trapped, he dispersed and formed a pair bond with 462 to form the Boulder Creek pack. Therefore, those two wolves produced pups in two different packs, but only after dispersal from their original pack. We found no evidence of males producing offspring simultaneously in more than one pack, and found no evidence that pups in any pack were sired by males other than the primary male of that pack.

Discussion

We used exclusion for molecular parentage analysis among radio marked wolf packs. The exclusion method uses genetic incompatibilities (i.e.mismatches of alleles) to reject parent – offspring hypothesis. Perfect exclusion can be difficult to attain if genetic variability within the sample is too low, if too few loci are genotyped, or if the pool of candidate parents contains siblings. Human error in genotyping, naturally occurring mutations, and null alleles also may introduce uncertainty into the exclusion parentage analysis (Jones and Arden 2003).

Our review of early studies of wolf genetic variability suggested that parentage analysis would be difficult in wolves because of relatively low heterozygosity and few alleles per locus. Heterozygosity is the sum of the frequencies of heterozygous genotypes at a given locus and is the most commonly used measure of genetic diversity. The heterozygosity over a number of loci is the mean of heterozygosities of individual loci. (Chambers 1983). Among 3 different populations in southern Canada heterozygosity (H_o) ranged from 0.58-0.63 with 4.4-4.5 alleles per locus in 10 loci genotypes (Forbes and Boyd 1997). However, the 22 new markers developed specifically for this study by WGI revealed a substantially higher level of genetic diversity (H_o=0.76,) than reported by Forbes and Boyd (1997)

That high level of heterozygosity and the large number of loci analyzed allowed clear parentage discrimination in our sample. In 65 cases a candidate offspring's genotype matched at all loci (i.e. 0 mismatches) with only a single putative mother-father pair. In one case a 22 loci match was found for a single mother but 2 males were candidate fathers. Investigation of the field data

for the candidate males showed that one was a known aged wolf born the year prior to the putative offspring, thereby excluding that male as a parent and identifying it as an older sibling.

Although a single mismatch is technically sufficient to exclude a parent-offspring hypothesis, errors in genotyping, or a mutation, could result in a single mismatch score from a true parent-offspring relationship. Our sample contained single mismatches (i.e. matches at 21 of 22 markers) for 10 sets of candidate offspring- parents. We used age data and field observation data to confirm that in 8 of those cases, the parents of the candidate offspring had already been identified with perfect 22 loci matches and the 21 loci match reflected a candidate pairing between the offspring's father and a full sister. In the other two cases data on relative age clearly identified the relationships as siblings or as an offspring being identified as a potential parent to its known parent. Therefore, the single mismatches were totally explained and did not represent parent-offspring relationships, further supporting our assumption that the 66 perfect matches represented true offspring-parent relationships.

Conclusions and Management Implications

Previous work on wolves indicated low heterozygosity, but our results indicate that parentage analysis is possible with 22 locus gentotypes. We found no evidence of multiple paternity within packs but multiple dams were identified in 2 packs. Previous studies using ultrasound for pregnancy diagnoses indicated that multiple litters were common in this population (McNay et al. 2006). Our genetic data revealed that primary males breed non daughter secondary females that are daughters of the current primary female and those females produce surviving pups. Pack social structure therefore contributes to multiple littering. A change in the alpha male within an established pack immediately changes the status of secondary females from daughter to non daughters making them eligible for breeding. Turn over among primary males may occur through natural mortality or by exploitation by humans. Low exploitation rates by hunting and trapping therefore may contribute to multiple litters if alpha males are removed and other pack members remain. This change in social structure could conceivably increase reproductive output of a wolf population sufficient to offset population declines from human exploitation.

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Table 1. Characteristics of 22 DNA microsatellite markers chosen for parentage analysis of wolves in GMU 20A, Alaska.

Marker	Chromosome.	Repeat Sequence.	Number of Alleles	Heterozygosity
C01.251	01	$(CA)_{17}$	6	0.88
C02.030	02	$(GT)_4(CT)_{13}$	8	0.60
REN233H01	03	$(CA)_{19}$	5	0.77
REN144A06	04	$(CA)_{19}$	6	0.81
REN69B24	07	$(CA)_{18}$	4	0.73
REN68B08	10	$(CA)_{22}$	6	0.89
REN105L03	11	$(CA)_{26}$	5	0.62
AHT121	13	$(CA)_{28}$	8	0.89
CPH9	29	$(GT)_{18}$	5	0.77
REN145P07	09	$(CA)_{21}$	6	0.73
REN262I12	12	$(CA)_{24}$	5	0.77
REN66E15	15	$(CA)_{19}$	6	0.92
REN85N14	16	$(CA)_{20}$	4	0.73
REN112G06	17	$(CA)_{22}$	6	0.85
REN183B03	18	$(CA)_{19}$	6	0.77
REN297N05	19	$(CA)_{21}$	5	0.73
REN316E23	20	$(CA)_{20}$	6	0.69
REN199O08	21	$(CA)_{18}$	5	0.77
REN210D03	23	$(CA)_{19}$	4	0.42
REN106I06	24	$(CA)_{21}$	7	0.88
REN94H15	25	$(CA)_{19}$	6	0.56
REN181L14	27	$(CA)_{20}$	5	0.85

Pack of Capture for Offspring	Male Parent	Female Parent	Offspring
2	02-294	02-139	295,325
45	02-294	02-139	331
4	04-148	04-149	195,196,298,299,312,313,314,315, 316,317,318,319,365,367
5	05-150	05-152	192,193
46	46-150	46-362	350,351,352,357,358,363
33	33-153	33-265	270
7	07-156	07-199	155,157,158,184,185,186,302,303
50	07-156	07-199	353
7	07-156	46-362	187
38	38-284	38-285	286,327,339
8	08-159	08-160	183,343,344,345,348

8	08-159	08-161	181,347,349
8	08-159	08-162	329,346,
8	08-159	08-179	180,182
14	14-175	14-272	297
17	17-169	17-170	307,309,311
17	17-169	17-200	166,167,168,308,310,330,332
31	31-322	31-338	173,320,323,369
Totals	10 Males	15 Females	66 Offspring
		in 11 packs	

XI. PREPARED BY: **APPROVED BY:**

Mark McNay   _____
Wildlife Biologist III   Tom Paul
                                                    Federal Aid Coordinator
                                                    Division of Wildlife Conservation

SUBMITTED BY:

Mark McNay   _____
Research Coordinator   Matt Robus, Director
                                                    Division of Wildlife Conservation

                                                    APPROVAL DATE: _____