Journal of Mammalogy, 87(1):85–92, 2006

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 examined 68 adult female wolves with ultrasound during late March and early April to diagnose pregnancy and litter size. Seventy-two percent were pregnant. We compared ultrasound diagnoses with postmortem embryo or placental scar counts in 14 females that died within 10 months of 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. In the postmortem sample we detected a decline in average placental scar color density between mid-September and mid-February. 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.

Key words: Alaska, *Canis lupus*, denning, fetal growth, litter size, placental scars, pregnancy, ultrasound, wolf

Pup production and survival are the most significant factors contributing to gray 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- to 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).

Causes and extent of early pup mortality remain generally unknown (Fuller et al. 2003) because litters are concealed within dens for approximately 3 weeks after birth (Mech 1970). After wolf pups emerge from the den, direct observations may not confirm parentage because all reproductive-aged wolves of both sexes participate in the digging of dens and in the feeding and protection of pups (Mech et al. 1996). Also, litter size can be overestimated and pregnancy rates underestimated because litters from multiple adult females within a pack are often consolidated into a single group during the denning period or shortly thereafter (Mech and Boitani 2003; Mech et al. 1998; Murie 1944).

We used portable ultrasound equipment to investigate reproductive parameters of wolves as part of a larger study on numerical and social dynamics of an exploited wolf population. Ultrasound is a common veterinary tool in the diagnosis of pregnancy among domestic and zoo animals. It

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has been used to estimate litter size in snowshoe hares (*Lepus americanus*) captured from the wild (Griffin et al. 2003) and for pregnancy determination in various free-ranging ungulates, including bighorn sheep (*Ovis canadensis*—Drew et al. 2001), moose (*Alces alces*—Stephenson et al. 1995), mule deer (*Odocoileus hemionus*—Smith and Lindzey 1982), and pronghorns (*Antilocapra americana*—Canon et al. 1997). Here, we evaluate the accuracy of ultrasound, relative to postmortem evidence, in the diagnosis of pregnancy and litter size in wolves. We also present fetal growth regressions calculated from our ultrasound measurements of wolves and compare those with models of fetal growth reported for domestic dogs.

Materials and Methods

Study area.—Our 11,500-km^2 study area in central Alaska was centered approximately 80 km south (64°10′N, 147°45′W) of Fairbanks. The climate is continental; daily temperatures average −23°C in January and 17°C in July. Elevations range from 300 to 4,000 m, sloping upward from poorly drained flats of boreal spruce–birch forest (*Picea* and *Betula*), through a foothill zone of alpine shrubs (*Salix*, *Alnus*, *Betula*, and *Populus*) and tundra sedges (*Carex* and *Eriophorum*), to the crest of the Alaska Range mountains. The terrain above 2,000 m is covered by large areas of permanent snow or glacial ice and supports little vegetation.

Ungulate prey species included moose, caribou (*Rangifer tarandus*), and Dall's sheep (*Ovis dalli*). Moose were the most abundant (approximately 800 moose/1,000 km^2—Boertje et al. 1996) and the primary prey of wolves (McNay and Ver Hoef 2001). Autumn wolf densities ranged from 15 to 19 wolves/1,000 km^2 between 1995 and 1998 (Valkenburg et al. 2004).

Ultrasound examination.—Wolves breed from late February through mid-March in interior Alaska; young are born from late April through mid-May. We captured wolves for ultrasound examination between 29 March and 12 April 1996–1999. Wolves were captured by darting from a helicopter using 3-cc Cap-Chur darts (Palmer Cap-Chur Equipment, Douglasville, Georgia) loaded with 500–560 mg of Telazol (tiletamine HCl and zolazepam HCl, Fort Dodge Lab, Fort Dodge, Iowa) and propelled by low-velocity charges. Each wolf was fitted with a mortality-sensing radiocollar (Telonics, Mesa, Arizona). Wolves remained immobilized for 1–3 h after induction of the initial dose; we injected an additional 50–100 mg of Telazol only if wolves regained sufficient motor function to interfere with ultrasound examinations. Humane capture and handling procedures were consistent with those established by the Alaska Department of Fish and Game Animal Care and Use Committee and conformed to recommendations of the American Society of Mammalogists (Animal Care and Use Committee 1998).

Captured female wolves were placed in dorsal recumbancy and scanned with an Aloka 210 portable ultrasound using a 5-MHz transducer (Aloka, Wallingford, Connecticut). We shaved a Y-shaped patch of hair from the inguinal area corresponding to the position of the uterine horns and coated the skin with vegetable oil to provide continuous contact with the transducer. We began the scan near the pubic bone using the anechoic urinary bladder as a landmark and made multiple scans along each uterine horn to identify and count individual embryonic vesicles. Using electronic calipers, we measured crown–rump length (CRL) of fetuses in a straight line from the most posterior point of the rump to the most anterior point of the crown when fetal structure and clarity of the image were sufficient to distinguish the head and developing body mass. If the vesicle was oblong, we measured embryonic vesicle diameter (EVD) as the maximum width (i.e., perpendicular to the longest axis) of the chorionic cavity. If the vesicle was spherical, EVD was measured as the diameter of the sphere. We recorded both CRL and EVD for a vesicle if image clarity, fetal structure, and fetal position were adequate. We measured 1–5 embryonic vesicles within each pregnant female, but averaged those measurements to produce 1 EVD and 1 CRL value for each pregnancy. Some females were examined by ultrasound during 2 or 3 consecutive years; we treated each annual examination as an independent observation of pregnancy and litter size.

Gestational age.—After ultrasound examination we monitored wolf movements and denning activities from fixed-wing aircraft, attempting 3 flights each week from 15 April to 27 May. We estimated parturition date as the 1st day on which a female entered a den and then remained confined to that den for $\geq$2 subsequent radiolocations. Weather and logistic constraints prevented location of all potential denning females each flight, therefore time between the 1st location at the den and the previous location ranged from 1 to 6 days ($\bar{X} = 2.8$ days $\pm$ 0.25 *SE*). When the interval between den entry and the previous location was more than 1 day we assumed den entry occurred on the temporal midpoint between the 2 locations. Eighty percent of the intervals between confirmed den entry and the previous location were $\leq$3 days; the remainder were 4–6 days. The greatest potential error in estimating den entry date was therefore 3 days. The average of all potential maximum errors was 1.4 days.

In wolves and dogs, duration of pregnancy is 60–65 days, and a surge in blood serum levels of luteinizing hormone (LH) occurs approximately 2 days before ovulation (Concannon et al. 1983; Seal et al. 1979). Using the estimated parturition date, we calculated gestational age at the time of ultrasound examination by assuming the LH surge (day 0) occurred 65 days before parturition. That is consistent with calculations of gestational age reported for dogs (England and Allen 1990; Kutzler et al. 2003; Yeager and Concannon 1990).

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

Postmortem examination.—We avoided investigation of den sites during the postparturition denning period to preclude research effects on den site fidelity and pup mortality. We could not compare our ultrasound estimates with the number of live-birth pups because the extent of neonatal mortality before den emergence was unknown. However, hunters and trappers legally killed wolves in our study area each year from August through April; that harvest included several of our research animals. Therefore, we evaluated the accuracy of ultrasound diagnoses using a postmortem sample of wolves that had been examined by ultrasound the previous spring. When females died before giving birth we obtained in utero fetal counts directly; otherwise we used placental scar counts (Englund 1970; Lindstrom 1981, 1994) to confirm pregnancy status and estimate in utero litter size.

From the postmortem sample, we removed reproductive tracts from all females older than 10 months, soaked those tracts in water for at

least 2 h to remove superficial blood, and then dissected and examined each uterine horn for embryos or placental scars. We graded placental scars among 6 categories of color density from faintly visible (1) to solid black (6—Englund 1970). Current-year scars were estimated as the number of scars (n_d) in the darkest shade category (d) plus the number (n_{d-1}) in the next lighter shade category (d − 1). Scars that were more than 1 shade lighter than the darkest scar in a given reproductive tract were not counted. We calculated a weighted mean scar shade for each reproductive tract as

$$\frac{(d)(n_d) + (d-1)(n_{d-1})}{(n_d + n_{d-1})}$$

Data analysis.—To illustrate the fetal growth curve, we used least-squares regression (Sokal and Rohlf 1981) with fetal measurements of EVD and CRL as response variables on the independent variable gestational age. We regressed gestational age as the response variable on fetal measurements to calculate estimates of gestational age and predicted birth dates. To evaluate whether timing of our ultrasound examinations affected accuracy of gestational age prediction, we regressed observed gestational age on the residuals of individual observations from the EVD and CRL regressions.

To examine the relationship between scar shading and time since parturition, we regressed mean scar shade on days since parturition using only samples with a known (within 1 week) date of death. Time since parturition was calculated as the difference between date of death and the population's mean parturition date of 7 May because precise birth dates were not known for each individual in that sample.

We used contingency table analysis to test differences in pregnancy rates between ultrasound and postmortem samples, and *t*-tests to test differences between mean litter sizes in ultrasound and postmortem samples (Sokal and Rohlf 1981). We used Kruskal–Wallis 1-way analysis of variance by ranks (Daniel 1978) to evaluate if the distribution of bias in estimates of litter size was different between our ultrasound data and those of other studies. We compared the proportion of failed litters observed in our study with failure rate observed in wolves within Denali National Park using contingency table analysis and evaluated the difference in mean litter size between the 2 populations using a *t*-test (Sokal and Rohlf 1981).

Results

Pregnancy and litter size.—We conducted 68 ultrasound examinations of 44 different adult (i.e., ≥22 months) female wolves from 36 annual packs during 1996–1999 and examined reproductive tracts from 46 additional adult females killed by trappers between autumn 1993 and spring 2001. No difference was found in pregnancy rates between the ultrasound (72%) and postmortem samples (63%; $\chi^2 = 1.03$, $d.f. = 1$, $P = 0.4$). We obtained a postmortem estimate of pregnancy from 14 adult females that died within 10 months after ultrasound examination and found 100% agreement in pregnancy diagnoses (12 pregnant and 2 not pregnant). None of the 10- to 12-month-old females scanned by ultrasound ($n = 3$) or examined postmortem ($n = 11$) were pregnant; nor did we find placental scars in the reproductive tracts of 38 yearling wolves (17–21 months) killed by trappers and hunters during winters 1993–2000.

We found no significant difference between in utero litter size via ultrasound of live-captured pregnant females during 1996–1999 ($\bar{X} = 5.1$, $n = 49$) and that determined by placental scars in other postparturient females ($\bar{X} = 5.6$, $n = 29$) killed by hunters and trappers between 1993 and 2001 ($t = 1.99$, $P = 0.25$). Counts in both samples varied from 1 to 9 fetuses per litter.

Table 1.—In utero estimates of litter size from ultrasound and postmortem counts obtained from 14 female wolves that died within 10 months after examination by ultrasound.

Wolf ID	Month of death	Ultrasound count			Postmortem count			Difference
		Left[a]	Right	Total	Left[a]	Right	Total	
137	December	2	1	3	2	1	3	0
139	February	4	2	6	4	2	6	0
149	January	3	2	5	3	2	5	0
155	February	0	0	0	0	0	0	0
161	January	5	2	7	3	3	6	1
168	December	0	0	0	0	0	0	0
190	September	2	1	3	3	1	4	−1
199	January	3	3	6	4	4	8	−2
200	September	4	5	9	3	5	8	1
201	February	2	1	3	3	1	4	−1
253	April	4	3	7	4	3	7[b]	0
265	October	4	3	7	4	3	7	0
295	April	3	2	5	2	2	4[b]	1
297	April	—[c]	3	3	—[c]	3	3[b]	0
Total		36	28	64	35	30	65	−1

[a] Left and Right refer to the left and right horns of the uterus.
[b] Postmortem count of fetuses in preparturient females; all other postmortem counts in this table were placental scar counts.
[c] Left horn of reproductive tract destroyed by scavengers postmortem; 3 fetuses were identified in each uterine horn by ultrasound.

In our paired sample of 12 pregnant wolves, 6 ultrasound counts matched postmortem counts exactly, 11 were within 1 fetus of postmortem counts, and all were within 2 fetuses of postmortem counts (Table 1). Mean bias was −0.08 fetuses per litter ± 0.26 *SE* in ultrasound counts relative to postmortem counts. Mean bias from 2 other studies of ultrasound diagnoses in dogs (England and Allen 1990; Toal et al. 1986) and from 1 study of snowshoe hares (Griffin et al. 2003) were similar, ranging from −0.23 to −1.19 young per litter. We found no difference in distributions between studies ($\chi^2 = 3.59$, $d.f. = 3$, $P = 0.31$).

The shading of placental scars was lighter in wolves killed in late winter compared to autumn samples; samples from mid-winter showed the greatest variability in mean scar shading (Fig. 1). Among most pregnant females examined postmortem we found light or incomplete scars that we interpreted as either remnants from previous years or current-year resorptions or abortions (Table 2).

Denning.—Among 49 adult females diagnosed as pregnant by ultrasound, 3 died before the denning period and 7 (15.2%) failed to den; 6 of those 7 were 2 years old. Two additional females (4.4%), age 2 and 3 years, entered and then abandoned dens within 1 week. The remaining 37 (80.4%) pregnant females, including 6 (46.1%) of the 13 pregnant 2 year olds, remained at dens throughout the denning period. All but 2 of those 37 were subsequently observed with pups during summer. We continued to monitor 18 adult females diagnosed by ultrasound as nonpregnant; none established dens.

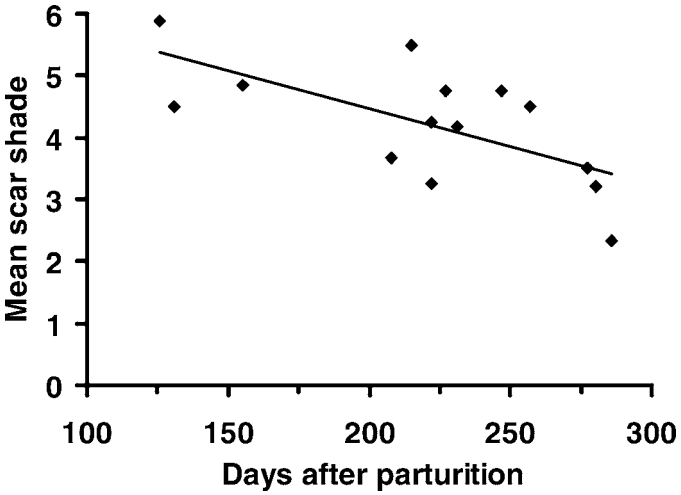

FIG. 1.—Color shade of current-year placental scars regressed on days after the mean population parturition date of 7 May ($r^2 = 0.43$, $P = 0.01$). For each reproductive tract, scar color shade was calculated as the mean weighted value of placental scars in the darkest category and in the category 1 shade lighter. Color shades ranged from 1 (faintly visible) to 6 (solid black).

Pregnant females entered dens from 27 April to 16 May with a mean of 7 May; some females briefly visited dens as early as 13 days before den entry. Radiotelemetry locations of pregnant females tended to be closer to dens as parturition approached, but few females localized at dens in the final days before parturition (Fig. 2). Distances from the den ($n = 21$) on the day before den entry ranged from 0.9 to 11.6 km ($\bar{X} = 4.8$ km $\pm$ 0.68 *SE*).

TABLE 2.—Distribution of placental scars among shade classes for 14 females with a known date of death. High number of total placental scars relative to estimated in utero litter size reflects remnant scars from previous pregnancies, residual scars from current-term resorptions or abortions, or both. Placental scar shades varied from faintly visible (1) to solid black (6).

Wolf ID	Month of death	Counts among placental scar shade classes 1–6						$\bar{X}$ scar shade[a]	Total number of placental scars	Estimated in utero litter size[b]
		1	2	3	4	5	6			
190	September	2	1	3	2	2	0	4.5	10	4
200	September	0	4	2	2	1	7	5.9	16	8
265	October	1	1	3	1	6	0	4.9	12	7
278	December	2	3	3	1	0	0	3.2	9	4
137	December	0	0	1	2	0	0	3.7	3	3
334	December	1	0	1	3	1	0	4.2	6	4
173	December	4	4	1	5	1	0	4.2	15	6
162	December	2	4	1	1	3	0	4.8	11	4
152	December	1	4	2	1	4	4	5.5	16	8
4211	January	0	2	0	2	2	0	4.5	6	4
199	January	1	2	1	2	6	0	4.8	12	8
139	February	1	4	2	0	0	0	2.3	7	6
327	February	0	1	4	1	0	0	3.2	6	5
201	February	0	1	2	2	0	0	3.5	5	4

[a] Weighted mean value for 2 darkest shade classes represented.

[b] Sum of placental scar counts in 2 darkest shade classes within each reproductive tract.

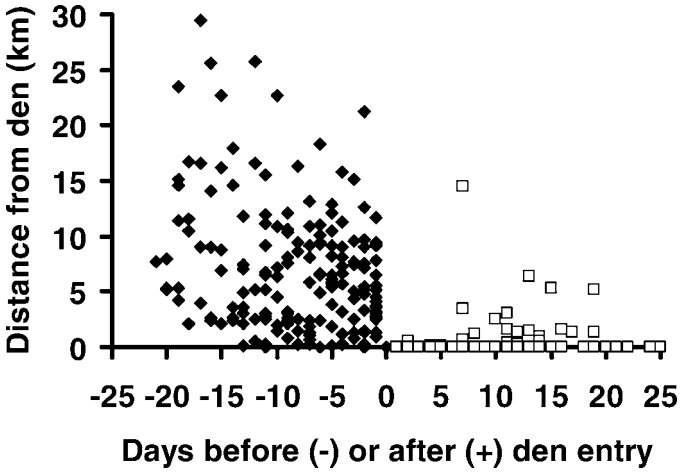

FIG. 2.—Straight-line distance of 35 radiocollared pregnant female wolves from their den sites during 3 weeks before (◆, $n = 175$) and 3 weeks after (□, $n = 225$) den entry.

Fetal growth.—Assuming parturition occurred 65 days after the LH surge (Concannon et al. 1983) and that parturition date coincided with den entry, our ultrasound examinations occurred between 24 and 42 days after the LH surge. Fetal measurements were recorded from 23 pregnancies. We measured CRL in 11 pregnancies and EVD in 19 pregnancies. CRL was a better predictor ($r^2 = 0.92$) of gestational age (y) than EVD ($r^2 = 0.79$). In our CRL model ($y = 6.79(\ln)x + 27.45$), 73% and 91% of observed den dates were within 1 and 2 days, respectively, of model predictions. In the EVD model ($y = 4.19x + 21.06$), 58% and 74% of observed den dates were within 1 and 2 days of model predictions. No relationship was found between examination timing and error in gestational age prediction in either the EVD ($r^2 = 0.07$, $P = 0.29$) or CRL ($r^2 = 0.19$, $P = 0.18$) models.

Embryonic measurements in both our wolf models were greater than predicted by Yeager et al. (1992) for beagle dogs. For giant-breed dogs >40 kg, Kutzler et al. (2003) recommended subtracting 2 days from the gestational age predicted by the beagle regression to reflect the larger fetal size in giant breeds. Body mass of denning females in our study averaged 40.0 kg $\pm$ 0.81 *SE* and CRL growth in our wolves closely matched that predicted for giant dogs (Fig. 3).

Wolf EVDs were also similar to those expected in giant dogs (Fig. 3), but our wolf EVD measurements were more variable ($r^2 = 0.79$) than reported for dogs ($r^2 = 0.94$; Yeager et al. 1992). Although some variability in both our models likely resulted from error in estimating parturition dates, we observed elongated vesicles in our earliest ultrasound examinations (24 days after the LH surge) and believe variability in the EVD model was primarily related to measurement error of nonspherical vesicles.

Safety of ultrasound.—One female died during capture when she suffocated in deep powder snow before we could reach her. Another female died 4 days after her capture approximately 5 km from her capture site, but cause of death was unknown. Attributing both of those deaths to capture, our research-induced

mortality rate was 2.8% among the 71 captures during our ultrasound study. That rate is similar to the mortality of 2.6% we experienced among 195 additional captures for other aspects of the study, and to the 2.3% mortality rate experienced during 350 captures in a separate study of the adjacent Denali National Park wolf population (L. Adams, United States Geological Survey, pers. comm.).

No evidence was found that capture during midgestation using Telazol affected denning or productivity. We captured pregnant females during the 2nd trimester of pregnancy in 36 annual packs. The failure rate to produce pups among packs examined by ultrasound (17%), was similar to the rate reported by Mech et al. 1998 (15%, $n = 91$ annual packs, $\chi^2 = 0.03$, $d.f. = 1$, $P = 0.86$) in Denali National Park where wolves were not captured during the 2nd trimester of pregnancy. Our mean autumn pup count (4.7 pups per pack) among packs examined by ultrasound ($n = 36$) was higher than autumn pup counts (3.8 pups per pack) in the Denali sample ($n = 91$, $t = 1.66$, $P = 0.13$).

Discussion

Pregnancy and litter size.—Radiography is the most accurate method for pregnancy detection in dogs, but is only useful late in pregnancy (>45 days after the LH surge) when the skeletal structure of the fetus has mineralized (Mattoon and Nyland 1995). In contrast, free-floating blastocysts have been detected by ultrasound 10 days after breeding (Cartee and Rowles 1984). Implantation of the anechogenic canine blastocyst occurs about day 20 after the LH surge and development into an embryo with an echogenic internal structure occurs at about days 23–25 (Yeager and Concannon 1990).

Our ultrasound examinations of wolves occurred between 24 and 42 days after the LH surge and we found 100% agreement in pregnancy diagnoses between ultrasound and postmortem examinations. Complete accuracy in pregnancy diagnoses has been reported in dogs (Bondestam et al. 1983; Toal et al. 1986) during the 2nd and 3rd trimesters of pregnancy, but in other studies, some pregnancies were not detected early in the 2nd trimester (England and Allen 1990; Shille and Gontarek 1985). England and Allen (1990) reported that greatest accuracy in pregnancy diagnoses in dogs weighing 22–30 kg was achieved beginning the 25th day after the LH surge. However, because wolf embryos are larger, they are more easily detected at an earlier age. We believe accurate pregnancy diagnoses of wolves could be achieved at 21 days after the LH surge, when embryonic vesicle diameters are approximately 0.5 cm. Vesicle diameters as small as 0.2 cm have been consistently measured in dogs (Yeager et al. 1992).

Transducer frequency may affect diagnosis accuracy. Higher frequencies provide greater resolution and greater potential for detecting small blastocysts earlier in the pregnancy (Yeager and Concannon 1990). Lower frequencies provide deeper sound wave penetration (Nyland et al. 1995) and therefore are more suitable in larger animals. Pregnancy diagnoses in dogs have been reported with the use of transducers ranging from 2.4 to 10 MHz (Bondestam et al. 1983; England et al. 1990; Mattoon and Nyland 1995; Yeager and Concannon 1990). Our results suggest that the 5-MHz transducer used in this study was adequate in both resolution and penetration to successfully diagnose pregnancy and litter size in wolves.

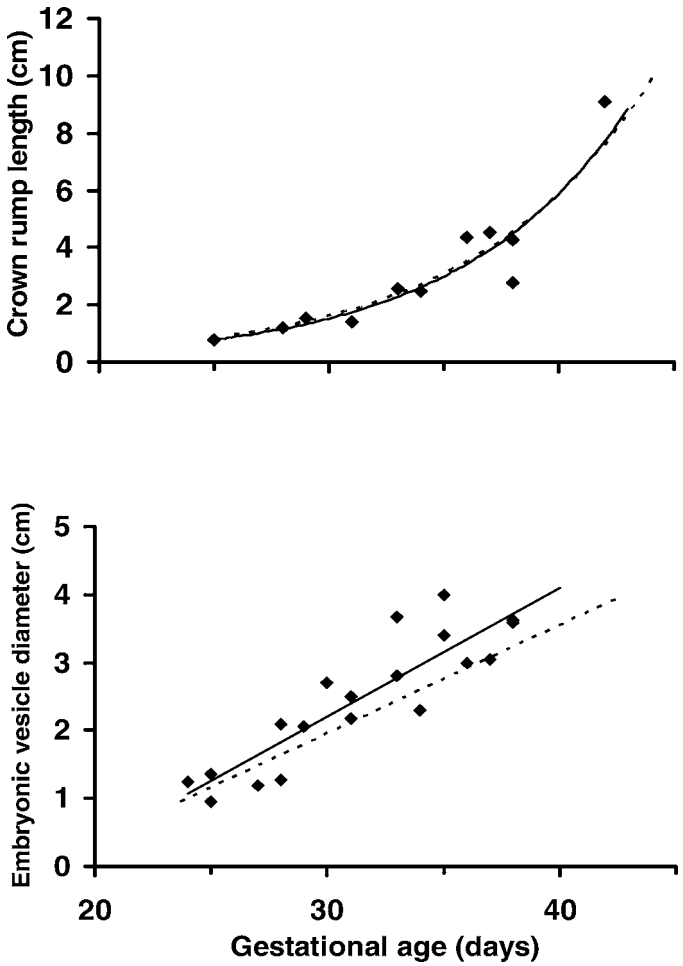

Fig. 3.—Comparison of wolf and giant dog regressions of (top) crown–rump length and (bottom) embryonic vesicle diameter on gestational age. Solid line is regression of wolf ultrasound data (◆) from this study (CRL = $0.026e^{0.135x}$, $r^2 = 0.92$; EVD = 0.19x − 3.48, $r^2 = 0.79$). Dashed line is regression of beagle dog ultrasound data calculated from Yeager et al. (1992) with a correction applied for giant-breed dogs (i.e., −2 days—Kutzler et al. 2003).

During our examinations we used the urinary bladder as a landmark to orient the ultrasound scan. A distended bladder can enhance visualization of the uterus in the pelvic region because it serves as a nonechogenic window, or it may confound examination if it displaces the uterus to either side of the midline; an empty bladder impairs visualization of the pelvic inlet (Mattoon and Nyland 1995). Reverberation artifacts and gas-filled bowel loops may be misidentified as, or conceal, embryos (Bondestam et al. 1983; Mattoon and Nyland 1995), and after day 35, variable fetal postures and overlapping fetuses caused by convolutions in the uterine horn can lead to error in fetal counts (England et al. 1990; Toal et al. 1986). Structural obstacles or misalignment probably contributed to errors in some of our fetal counts. In 1 case (wolf 161; Table 1) we misidentified the correct position of embryos in the right versus left uterine horn,

and in a 2nd case (wolf 199; Table 1) apparently failed to detect vesicles that were positioned in the caudal extremities of the uterine horns.

Litter size determination in dogs was reported to be most accurate between days 28 and 35 (Bondestam et al. 1983) and when litter size was 5 or less (Shille and Gontarek 1985; Toal et al. 1986). We expected higher accuracy of litter size detection in wolves than reported in dogs because wolves have larger embryos and smaller average litter sizes. Our findings support that hypothesis. Relative to placental scars, 50% of our litter size estimates from ultrasound counts were accurate, and 92% of our ultrasound counts estimated litter size correctly to within 1 fetus. In comparison, litter size diagnoses in dogs were reported as 31% accurate with 62–77% of in utero estimates correct to within 1 live-birth pup (England and Allen 1990; Toal et al. 1986).

Placental scars form after parturition when macrophages absorb residual blood at implantation sites then migrate into underlying tissue (Martin et al. 1976). Scars can persist for more than a year, but fade with time (Lindstrom 1981; Martin et al. 1976; Strand et al. 1995). Faded scars remain after resorption or abortion of fetuses (Englund 1970; Strand et al. 1995) and fetal resorption is common in some wolf populations (Hillis and Mallory 1996). Therefore, in multiparous females, the total placental scar count may represent current offspring, recently aborted or resorbed fetuses, and offspring from the previous year. Our sample included 1 primiparous female for which we had counts for both placental scar and ultrasound examinations (wolf 201; Tables 1 and 2); we counted 3 embryos with ultrasound, estimated 4 fetuses from dark placental scars, but observed a 5th faded, partial scar that likely represented a fetal resorption. Therefore, interpretation of placental scars can be problematic even among primiparous females.

Englund (1970) ranked the color darkness of placental scars in red fox (*Vulpes vulpes*) from 1 (barely visible) to 6 (solid black) in an attempt to distinguish scars representing current-year births from those of resorptions or previous-year births. Subsequent investigations in arctic fox (*Alopex lagopus*—Strand et al. 1995), red fox (Allen 1983; Lindstrom 1981, 1994) and Canada lynx (*Lynx canadensis*—Mowat et al. 1996) also used Englund's (1970) shading scale. Although Allen (1983) and Englund (1970) found general agreement between embryo counts and counts of only the darkest placental scars (shades 5 and 6) in red fox, Strand et al. (1995) found better agreement if category 3–6 scars were counted in arctic fox, and Mowat et al. (1996) recommended counting all color shades in lynx. Lindstrom (1981, 1994) advocated progressively adding lighter shades to the count as time after birth increased, reasoning that dark scars counted in samples shortly after parturition would have appeared as faded scars if the sample had been collected several months later.

Placental scars in red fox disappear completely in late proestrus (Fairley 1970) and our results suggest a similar pattern in wolves. Proliferation of the canine endometrium begins during proestrus and 1st occurs in the surface epithelium and underlying stroma (Van Cruchten et al. 2003). However, well before proestrus begins, the macrophages that comprise the visible placental scar have migrated beneath the stroma and are concentrated in the mesometrium (Martin et al. 1976). Therefore, the new endometrial tissue proliferates over existing placental scars during proestrus, gradually obscuring them from gross observation.

Proestrus in wolves lasts for 15.7 days $\pm$ 1.6 *SE*, and estrus persists for an additional 9.0 $\pm$ 1.2 days (Seal et al. 1979). Therefore, proliferation of endometrial tissue in wolves within our study area began in early February. Consequently, the darkest placental scars in our February samples had faded to class 3 or 4. Our latest February sample was killed on 18 February, but we recovered the reproductive tract from a female killed on 17 March that had been diagnosed by ultrasound as pregnant with 2 embryos the previous year. No scars were visible in the March sample; they were apparently obscured by the thickened endometrial lining. Therefore, we caution against counting placental scars during late proestrus, estrus, or pregnancy, a period lasting from late February to late May in our study area.

We found the best agreement between ultrasound and placental scar counts when we counted scars that were well spaced along the uterine horns and within 1 color shade of the darkest scar. We censored partial scars that did not traverse the entire width of the uterine horn, lighter scars that overlapped or abutted dark scars, and faded scars that were at least 2 shades lighter than other scars within that uterus. This method is similar to that proposed by Englund (1970) and Lindstrom (1981, 1994) but calibrates counts for both seasonal and individual variation in scar color density.

Denning and fetal growth.—Wolves may visit several potential den sites (Haber 1977; Thiel et al. 1997) and localize near their selected den before parturition (Harrington and Mech 1982; Packard 2003; Young 1944), but few females in our sample localized at dens before abruptly confining themselves to the den. In a captive wolf, Zimen (1981) noted that on the day of parturition the wolf's activity was normal in the morning, but in the afternoon the female entered the den and gave birth. Afterward she remained confined to her den for several days. We used those behavioral cues to establish parturition dates. Our movement data, and agreement of our fetal size regressions with those of giant-breed dogs, support our assumptions that den entry date was a reasonably accurate estimate of parturition date within our study area.

Differences in fetal size have been reported for various breeds of dogs; EVDs are greater in larger breeds at a given gestational age (Luvoni and Grioni 2000; Yeager et al. 1992). Although fetuses of giant dogs grow faster, there is no difference in gestation period between females of different body weight, nor are there effects of litter size on embryonic measurements (Kutzler et al. 2003). Examination of our data shows that fetal growth in wolves is similar to that of giant-breed dogs.

Yeager et al. (1992) reported that accuracy in estimating gestational age was similar between EVD and CRL in dogs, but noted that increases in the transverse diameter of the embryonic vesicle slows, or ceases altogether, for a period late in the 2nd trimester when CRL growth is most rapid. Therefore, CRL measurements can be made over a longer period (24–48 days

after the LH surge) compared to EVD (20–37 days after the LH surge), but accurately measuring CRL in the 3rd trimester becomes increasingly difficult because of crowding and flexion of fetuses (England et al. 1990; Yeager et al. 1992).

We found no relationship between accuracy in predicting parturition date and timing of ultrasound examinations between days 24 and 42 in either the EVD or CRL models. However in a large sample ($n = 83$) that included various dog breeds examined by ultrasound between days 20 and 62, Kutzler et al. (2003) reported the most accurate parturition date predictions from both fetal and embryonic vesicle measurements occurred at day 30 after the LH surge.

A spherical conceptus would yield a consistent measurement in any equatorial plane, but if the conceptus is elongated or flattened, consistent measurements become more difficult. England et al. (1990) reported that the conceptus in dogs remained spherical until 40 days after the LH surge, but others reported oblong embryonic vesicles (Yeager and Concannon 1990) or vesicles that were noncircular in the transverse plane (Kähn 1994; Luvoni and Grioni 2000; Yeager et al. 1992) between days 20 and 37. Our results indicate that elongation in the wolf embryonic vesicle can begin before day 24.

The lower precision of our EVD model probably reflected differences in shape between embryonic vesicles at a given gestational age and our failure to consistently measure in a precise transverse plane. We found it easier to measure CRL because the presence of a fetal structure allowed us to manipulate the transducer for a clear view of fetal length. Some embryos were not suitable for measurement because of the position of the uterine horns and limitations inherent in use of a single transducer frequency.

We found ultrasound to be safe and effective for diagnosing pregnancy and litter size in wild, free-ranging wolves. Risks to animal welfare arose from inherent aspects of field capture rather than from the ultrasound examination, and we found no deleterious influence on productivity or survival of pups after ultrasound examinations.

Acknowledgments

Many Alaskan trappers provided postmortem samples for this study; in particular we recognize the contributions of R. Perkins. Field assistance for wolf capture and radiotelemetry was provided by numerous field technicians from the Alaska Department of Fish and Game including L. Butler, T. Hollis, B. Scotton, and T. Seaton. T. Cambier, J. Larrivee, L. Larrivee, D. Miller, R. Swisher, and M. Webb safely piloted aircraft. M. Keech and D. Reed reviewed early drafts of the manuscript. J. Ver Hoef and D. Reed provided statistical advice. L. McCarthy provided technical editing. We sincerely thank them all. This study was funded by Federal Aid in Wildlife Restoration Grants and the Alaska Department of Fish and Game.

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Submitted 21 February 2005. Accepted 9 June 2005.

Associate Editor was Floyd W. Weckerly.