Estimating pregnancy rates and litter size in snowshoe hares using ultrasound

Paul C. Griffin, Leslie Bienen, Colin M. Gillin, and L. Scott Mills

Abstract Accurate estimates of pregnancy rate and litter size are essential to many studies of population dynamics. We examined the use of ultrasound for estimating pregnancy rate (the proportion of pregnant females in a population) and litter size and for monitoring fetal development and survival in snowshoe hares (*Lepus americanus*). We compared ultrasound-based estimates of fetal number in 16 females to either radiographic estimates of fetal number for the same females or to the number of newborn hares counted after parturition. With ultrasound we detected fetuses as small as 4 mm and at least 25 days before parturition. Ultrasound-based estimates of fetal number were correct for 8 hares, low by 1 fetus for 6 hares, and high by 1 fetus for 2 hares. Sequential ultrasound examinations detected change in fetal size and position, and detected ceased or abnormal fetal development in 2 hares. Ultrasonography is an effective, minimally invasive tool to monitor fetal number and pregnancy rate. Application of the technique to other species would require familiarity with internal anatomy and training in operation of the ultrasound machine.

Key words *Lepus americanus,* litter size, pregnancy rate, snowshoe hare, wildlife ultrasonography

Ultrasonography is widely used in zoo and traditional veterinary medicine but is less often used in wildlife field studies, despite its potential as a valuable tool. Because fat and intestinal contents are the main confounding factors when counting fetuses with ultrasound, estimates should be more accurate in wild animals than in pets or livestock because wild animals generally have less body fat. Obtaining an estimate of minimum fetal number in wild animals allows comparison of pregnancy rates and minimum litter sizes across different habitats, years, and management treatments.

Population dynamics of snowshoe hares (*Lepus americanus*) are the focus of recent attention in the southern part of their range because they are the primary prey of the Canada lynx (*Lynx canadensis*), listed as threatened in the contiguous 48 states (United States Fish and Wildlife Service 2000). Snowshoe hares give birth to as many as 4 litters per summer (Severaid 1942, Keith 1990). In their northern range, the number of offspring per female per breeding season varies from 7.5 in the decline phase of the cycle to 17.9 in the late increase phase (Cary and Keith 1979). Conception occurs immediately post-parturition (Severaid 1942, Bookhout 1964, Keith 1990) in a synchronous birth pulse (Hodges 2000*a*), with a gestation period of 34–37 days (Keith 1990).

Traditionally, litter sizes were estimated using methods such as abdominal palpation (Cary and Keith 1979), holding females in captivity until parturition (O'Donoghue and Krebs 1992), and necropsy of sacrificed pregnant females to count corpora lutea scars (corpora albicans; Bookhout 1964, Cary and Keith 1979). Each technique has advantages and disadvantages. Abdominal palpation requires no expensive equipment, and reliable detection of pregnancy with palpation has been

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reported 8 or 10 days after coitus (Bookhout 1964, Kieth et al. 1968), but we have not found publications validating its accuracy in assessing litter size in snowshoe hares. Palpation has the potential to damage or dislodge fetuses, particularly if performed at critical stages of placental development (Harkness and Wagner 1995); it requires a gentle technique (Short 1967), and early in gestation it can be difficult to distinguish fetuses from digestive organs (Tainturier 1988). Holding females in captivity for prolonged periods can produce significant, chronic stress levels, which can affect observed litter size at birth (Hillyer and Quesenberry 1997). Necropsy can not provide information about changes in fetal number or viability during the course of a pregnancy, is not acceptable in long-term studies at low population size when survival and density parameters are estimated from marked animals, and poses ethical conflicts. Radiography with X-rays yields precise estimates of litter size but is not practical for widespread diagnosis of pregnancy and fetal number unless gestational stage of the females is known because fetuses can only be visualized late in gestation, after the fetal skeleton has mineralized.

In domestic rabbits the use of ultrasound to diagnose pregnancy is now widespread (Cubberly et al. 1982, Inaba et al. 1986, Tainturier 1988, Beregi et al. 2000) and minimally invasive (Beregi et al. 2000), although it does require specialized equipment, training, and anesthesia. To complement long-term demographic studies of snowshoe hares in western Montana, we assessed the accuracy of abdominal ultrasound in determining litter size by comparing ultrasound estimates of fetal number to estimates using late-term radiographs and to counts of newborn hares born to females held in captivity for short periods of time.

Methods

In the summers of 2000 and 2001, we live-trapped adult female snowshoe hares in the Seeley Lake region of western Montana. We limited this research to the second and third birth pulses; litter size in the first birth pulse is consistently small (Keith 1990). We housed captive hares in 76 × 76 × 41-cm hutches on a forest litter substrate. We fed captive hares apple, common wild plants, and commercial pellets. We shaded cages with cloth and branches and hung an interior curtain for hare cover (Carol Stefan, University of British Columbia, personal communication). We released animals that met objective stress indications (weight loss, self-injury, hair pulling, or pacing) or subjective criteria (inappetence or aggression).

In 2000, we held 7 females in captivity 15–23 days, and held 3 captive at a forested site 6–9 days. Of these, we could use 6 to test ultrasound-based litter size estimates via comparison to X-ray radiography (5 hares) or necropsy (1 hare); we released the 4 others. In 2001, we held captive in forested sites 12 females that were identified as likely within 10 days of parturition via ultrasonography, until they gave birth; we released 2 of these after 7 days to minimize stress to the mother. Thus, sample size for evaluation of ultrasound was 16 hares for 2000 and 2001 combined.

We anesthetized hares prior to all ultrasound and radiologic exams. Five of 33 exams in 2000 were performed using isoflurane gas (Isoflurane USP, Schein Pharmaceutical, Phoenix, Ariz.) administered with a mask but no regulated delivery system; all other exams followed intramuscular injections of 9.4 mg/kg ketamine (Ketalar, Parke-Davis Pharmaceutical, Morris Plains, N.J.) and 1.75 mg/kg xylazine (Rompun, Bayer Pharmaceutical, New Haven, Conn.) given in the epaxial muscles. These low doses provided sedation for 30–50 minutes. We were concerned with potential effects of injectable drugs on fetal development, so in 2001 we used only regulated isoflurane gas. Isoflurane has the advantage over nonreversible injectable drugs in that when it is applied at low flow rates, the animal regains consciousness <5 minutes after the gas is turned off (Thurmonn et al. 1996). We placed each hare in an anesthesia box with 3–4% isoflurane flow rate until the hare was sedate, then applied a mask connected to a vaporizer and flow meter (Ohmeda, Madison, Wisc.; $900 used), maintaining 1–2 % flow of anesthetic gas.

We shaved anesthetized hares on the ventral abdomen from pubic bone to the most caudal rib prior to ultrasound examinations. We performed examinations with hares in dorsal recumbency, using a Universal SonoVet 600 veterinary ultrasound machine and a 6.5 MHz microconvex probe (Universal Medical Systems, Bedford Hills, N.Y.; $12,000 new). We obtained estimates of fetal number by counting separate echogenic masses inside the body and horns of the uterus (Figure 1), following each horn craniolaterally until the lumen was no longer visible. We measured each fetal mass in cross-section at its largest diameter by measuring

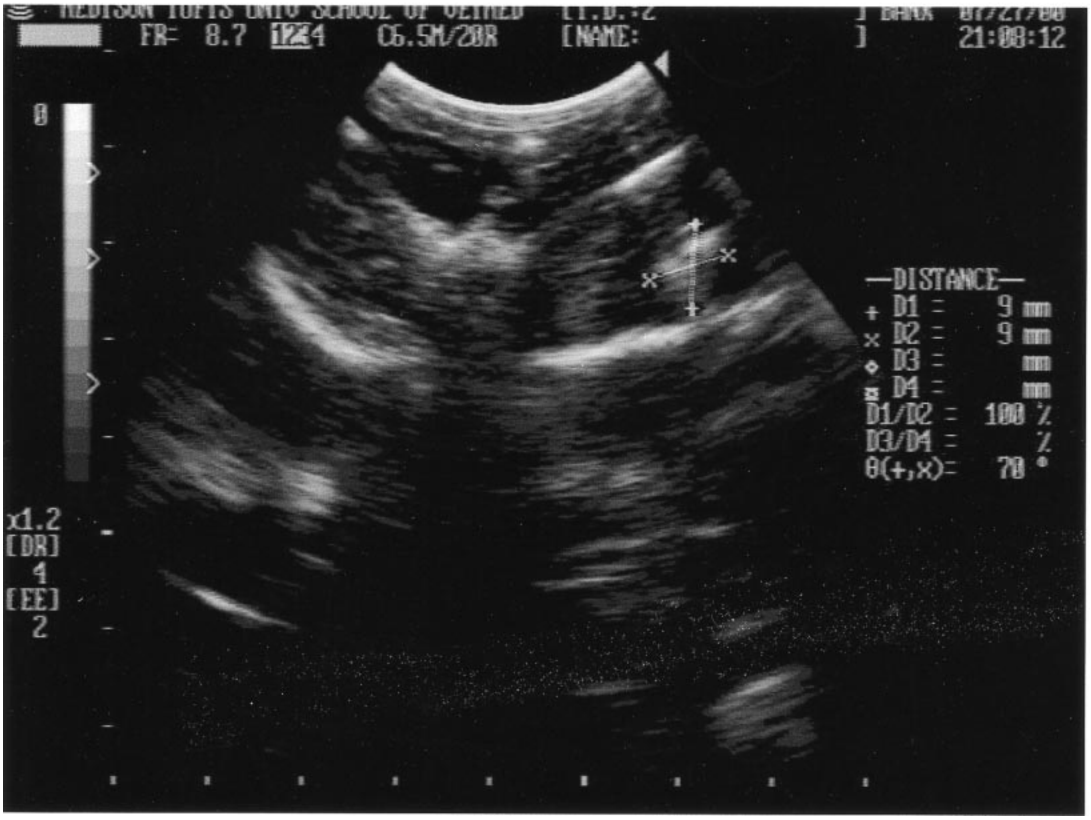

Figure 1. View from an ultrasound examination showing cross-sectional width of a fetus in the left uterine horn of a snowshoe hare. The measured diameters between both pairs of "+" cursors and "x" cursors were 9 mm.

from the dorsal edge of the fetus to the junction of the fetus and the uterine wall on the ventral aspect of the uterus. This method provided consistent measurements within each exam and allowed us to monitor fetal growth between ultrasound exams. Later in gestation we measured longitudinal spine lengths and cranial diameter of fetuses to estimate both fetal number and gestational stage (Figure 2). Only one ultrasound operator (L. B.) measured fetuses and estimated litter size in all examinations.

For ventrodorsal radiologic exams in 2000, we used high-speed, high-contrast film with a rare earth detail cassette and a MinXRay 803G portable X-ray machine (MinXRay Inc., Northbrook, Ill.; $3,200 used), at settings of 70kVp, 30mA, and 0.06 seconds with a focal distance of 76 cm. For 5 hares, radiographic estimates of fetal number were made in the radiology program at Tufts University School of Veterinary Medicine by counting individual fetal skulls. Fetal number also was determined by necropsy in a sixth hare in 2000 after she died, most likely due to unregulated isoflurane anesthesia.

To minimize the length of time females were held captive in 2001, we used ultrasound to approximate gestational stage of all females captured. Later-term fetuses were distinguished with ultrasound by their size and the relative development of skull, limbs, and vertebral column. Only females with very late-term fetuses were put into captivity. We recorded total litter size born to captive females within 24 hours of parturition. We esti-

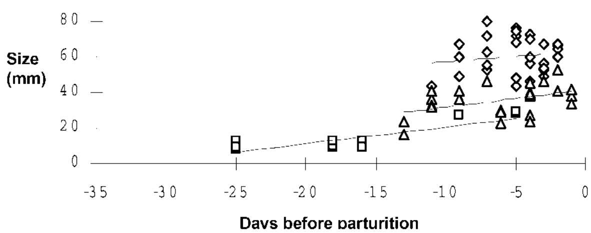

Figure 2. Three different measures of fetal size made with ultrasound (*y*-axis) are regressed against days before parturition (*x*-axis) for fetuses in 10 hares that gave birth in captivity. Squares and the lowest regression line represent cross-sectional diameter of individual embryos ($r^2 = 0.68$; $P = 0.00029$). Triangles and the middle line represent measures of head circumference ($r^2 = 0.02$; $P = 0.42$). Diamonds and the highest line represent measured spinal length ($r^2 = 0.19$; $P = 0.031$).

mated the earliest point in gestation when fetuses were visible based on females that gave birth in captivity and were examined early in pregnancy.

We assessed the bias of ultrasound-based litter size estimates by comparing ultrasound estimates to the observed number of fetuses visualized on late-term radiographs, counted after parturition or in necropsy. We examined one female that gave birth to 5 leverets with ultrasound twice, with ultrasound-based estimated litter sizes of 4 and 5; to be conservative when analyzing the accuracy of ultrasound as a technique, we used the incorrect ultrasound estimate.

We used Akaike's Information Criterion (AIC; Akaike 1973, 1974; Burnham and Anderson 1998) to compare 4 models against the data to assess fit. Models differed only in the expected values of litter size; observed litter size was the number of fetuses or newborns counted by radiography or after birth of the litter. Three models, "Accurate," "Negative bias," and "Positive bias," had expected values of litter size that were, respectively, the number seen on ultrasound examinations, one fetus higher, or one fetus lower. A fourth model, "Average," had only one expected value that was the mean litter size recorded for all hares in the study, using radiography or observed litter size at birth. The AIC value for each model was 2 × the number of parameters plus 2 × the negative log likelihood. We calculated model likelihood as a product of the likelihood of all 16 pairs of expected and observed values, given a Poisson distribution.

Results

We correctly determined the pregnancy status (pregnant or nonpregnant) of all females examined, as evidenced by fetal development detected with radiography or by parturition. We successfully detected fetuses of known gestational stages 25 days before parturition (9–12 days after coitus); these were 8, 9, 11, and 13 mm in cross-sectional diameter. The smallest measured fetus in a female of unknown gestation stage was 4 mm; by comparison, Bookhout's (1964) 9-day-old embryos were 3.5 mm on average in necropsy. Sibling fetuses measured with ultrasound varied in size, as has been noted in previous studies (Bookhout 1964). Growth curves for fetuses of known age (Figure 2) indicated that based on our small sample size, fetal cross-sectional diameter was a more reliable indicator of gestation stage early in pregnancy; this measure was also the most reliable of fetal age in small companion animals (Mattoon and Nyland 1995). Late in gestation, spinal length had a more predictable relationship to age than did head circumference.

Ultrasonography was a reliable technique for estimating minimum litter size; differences between estimated litter size and observed litter size did not exceed 1 fetus. Mean bias of ultrasonography for the 16 females was –0.375 because our predictions were low by 1 fetus for 8 hares, were correct for 6 hares, and were high by 1 fetus for 2 hares. The estimated bias was not the same at 3 realized litter sizes, but there was not a systematic pattern of greater bias at more extreme litter sizes. The average bias for litter size 3 was +0.4, at litter size 4 it was –0.71, and at litter size 5 it was –0.2. Practically, this means the most common mistake was for females actually carrying 4 fetuses; they often were estimated to have only 3 with ultrasound.

The best model to explain the relationship between our estimates and the observed values of litter size was one in which estimates equal litter size ("Accurate" model). A model in which our estimates were low by 1 fetus ("Negative bias") fit our data well, with differences in AIC (ΔAIC) of 0.0209. The model for litter size that used the mean of litter sizes counted by radiography or at birth ("Average") also fit the data well (ΔAIC = 1.54); models with ΔAIC < 2 are considered to have roughly comparable levels of parsimonious fit to the data (Burnham and Anderson 1998). The comparable fit of these 3 models to our data indicated that ultrasound was generally accurate or biased low by 1 fetus; simply guessing that each female had litter size identical to the average observed (via radiography, necropsy, or at birth) would have been comparably accurate to ultrasound. This last estimation procedure, though, presupposed knowledge about the average litter size (such data require some means of observation).

We obtained reliable estimates of fetal number from diagnostic-quality X-ray radiographs for 5 hares and from necropsy for 1 hare. In the necropsy true diameters of 3 fetuses were within 1.4 mm of diameters estimated by ultrasound, and their locations within the uterus corresponded to the positions estimated from the ultrasound exam. A fourth fetus present in the right uterine horn was not detected on ultrasound exam due to its extremely distal position, where it was more likely to be confused with intestinal contents.

Discussion

Ultrasonography can provide detailed, safe, and demographically useful information about the parameters of female fertility that potentially affect population dynamics of snowshoe hares. These parameters are pregnancy rate in a particular geographic area, habitat, or time span; the number of fetuses in early pregnancy; and the development of fetuses in late pregnancy.

All females with fetuses visible by radiology or evidenced by parturition were correctly diagnosed as pregnant by ultrasonography. Ultrasonography in particular is a good choice of technique if the pregnancy status or gestational stage of the animal is not known because ultrasound can reliably identify and measure fetuses at small sizes; in snowshoe hares we identified fetuses as small as 4 mm. Predictive growth curves for fetuses visualized with ultrasound (Figure 2) can be improved in the future as more studies record fetal size in litters with known parturition dates.

Measurements made with ultrasound can be used to identify late-term pregnant females, to minimize time in captivity until parturition. If parturition in rabbits is delayed 3 days beyond the mean gestation interval, fetuses can grow abnormally large and may die (Harkness and Wagner 1995). In 2001, 10 of 12 pregnant females identified as late-term gave live birth in captivity, with all 40 leverets born surviving to detection.

It is easier to determine pregnancy status of an animal than to count the precise fetal number. Identifying pregnancy rate alone answers some research questions. Interestingly, in 2001 we noted low pregnancy rates in the third birth pulse period across a large region of western Montana (Griffin, Bienen and Mills, unpublished data), a pattern that parallels pregnancy rates during the low phase of the 10-year cycle in the north (Cary and Keith 1979).

As long as ultrasonographic examinations are carried out by a trained individual, ultrasound-based estimates of litter size can be used as highly reliable estimates of the minimum values and reasonable estimates of true values. The "Accurate" and "Negative Bias" models both fit our data better than the "Positive Bias" model, suggesting that our ultrasound-based estimates were generally either correct or low by 1 fetus. Although overestimation of litter size can occur with ultrasonography, it was clearly not a source of systematic bias. The other model that had an equally good fit to the data, "Average," assumed that litter size was equal for all females and required accurate knowledge of the true value for mean litter size that could be accurately obtained only by widespread sacrifice or late-term radiography of many hares in a study site. Because litter size can vary so widely (Cary and Keith 1979), average values for litter size can not be assumed to stay constant among years or habitats. As a result, using the "Average" model would not allow for comparison of fecundity across birth pulses or habitats.

Identifying fetuses using ultrasound requires correct identification of the uterus and careful examination of the contents of the uterine horns from the caudal to cranial ends. For this reason, researchers applying ultrasound to any wildlife species must be familiar with the internal anatomy of their study organism and should be trained in the recognition of other echogenic structures that might be confused with fetuses, such as fecal material and kidneys. The training period for L. B. included 10 days of training in the ultrasound department of a university hospital with no particular emphasis on diagnosing pregnancy and 5 hours of training from an equipment salesperson. We expect the training period would be short for practicing veterinarians who have some experience with ultrasonography. For other users, veterinary schools and ultrasound machine manufacturers offer 2–3-day training courses in the use of ultrasound for abdominal examinations, including pregnancy diagnosis, for $800–1,500. Following such a course, the operator should practice with captive pregnant hares or rabbits until competency before use in the field.

Both ultrasonography and palpation can potentially be used to estimate litter size in animals captured at various stages of gestation, thus allowing for estimates of fetal resorption rates in animals captured and examined more than once. In contrast to palpation, ultrasound potentially provides more detailed information about gestational stage via precise measurement of fetal anatomical structures, verification of fetal viability by detecting heartbeats, and is considered less invasive. The high costs of equipment, the specialized training required to differentiate internal anatomical structures, and the need to administer anesthetics to pregnant animals are all disadvantages to the use of ultrasound. Ultimately, the choice of technique for a given study may depend on the level of acceptable risk to fetuses, the need for assessing the viability of fetuses counted, and the confidence of individual researchers in manual versus visual estimation.

In the southern part of snowshoe hare range, where habitat may be more varied than in the north (Howell 1923, Dolbeer and Clark 1975, Wolff 1980), the existence of a cycle is controversial (Hodges 2000*b*) and there is little information about litter-size variation across habitats or years. Along with more traditional methods, ultrasonography is one more tool that wildlife biologists could use to establish or refute the existence of regular cycles of fecundity corresponding to those observed in the northern range. The ability to compare litter sizes across habitats could help to identify the habitat features associated with high and low reproductive rates for a variety of wildlife species.

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