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Noninvasive sampling for carnivores

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Now is an exciting time to study carnivore ecology via noninvasive sampling methods. Technological and methodological advances, and new techniques for data analysis, have contributed to a rapid increase in noninvasive carnivore studies. These studies complement and extend inferences from traditional sampling regarding individuals, populations, and communities. Today, researchers can estimate size and survival rate for a population, estimate historic and current rates of movement across fragmented landscapes, and measure carnivore stress loads without ever catching, handling, or even seeing a single animal. Noninvasive sampling is the gathering of data without capturing, handling, or otherwise physically restraining individual animals. The techniques usually imply that a target animal is not observed during data collection and, presumably, is unaffected by data collection. Although direct animal observations for behavioral studies and for distance sampling may also be considered noninvasive, we do not include these direct observation methods. Noninvasive data-collection methods include sign surveys, diet analyses, camera trapping, DNA extraction, and endocrine (see Chapter 12) or disease monitoring (see Chapter 13) from scats and hair. Perhaps a better name is "less invasive" because we do not really know the impact, for example, of removing scat samples found in a jaguar (*Panthera onca*) habitat for 2 months. Such a study might disrupt marking behavior and unknown impacts could arise in a study site from the presence of a trained scent-detecting dog locating scats. Nonetheless, the term has gained familiarity and become conventional (Long *et al.* 2008a).

Why use noninvasive sampling? The advantages are numerous. Capture and handling are highly stressful and potentially dangerous to both humans and animals, especially with large carnivores. Invasive studies require more permitting, especially with endangered species, and often suffer issues with animal care and use

committees. In addition, capture, handling, and subsequent monitoring are usually expensive, logistically difficult, time consuming, and result in small samples sizes, limiting population-level inferences, especially for elusive, low-density, or trap-shy animals. By contrast, noninvasive techniques can produce larger sample sizes, reducing bias, increasing precision, and broadening the scope of potential hypotheses. Noninvasive field sampling is often relatively simple to employ and to standardize, training inexperienced people can be easy and studies can cover large areas. Finally, noninvasive sampling is less likely to induce a trap response in animals, again reducing human-induced bias.

While noninvasive techniques supply new information and hold great promise, we do not suggest that they should replace all traditional capture and handling studies, such as those to obtain information about body condition, to collect blood, or to affix transmitters for studies of movements, home ranges, and habitat selection (Chapters 6, 7, 8, 9, and 10). This chapter echoes and extends the recent book on this topic (Long *et al.* 2008a), and focuses on recent advances.

4.1 Methods of noninvasive sampling

4.1.1 Sign surveys

Naturalists have sampled carnivores noninvasively for decades. Skillful, field-based identification of tracks, scats, kills, bones, and hair have illuminated much of what we know about distribution and habits of carnivores, and have instilled a deep appreciation for natural history. In fact, identification of animal sign can be quite reliable in some instances. For example, Prugh and Ritland (2005) identified coyote (*Canis latrans*) scats by morphology with >90% accuracy in the Alaska Range, despite the presence of three other similarly sized carnivores. In other cases, however, scat identification by morphology alone is prone to error. For example, 18% of scats identified with high confidence by experienced field collectors as marten (*Martes martes*) scats were actually from foxes (*Vulpes vulpes*, Davison *et al.* 2002). Misidentification of carnivore sign in the field occurs more often when the target species is rare (Prugh and Ritland 2005).

As with scat, identification of tracks in snow, dirt, and mud can be useful and at times reliable. However, identification problems can arise due to substrate quality and animal movements (Heinemeyer *et al.* 2008). If concerns about uncertainty can be ameliorated, track surveys can be effective and inexpensive for occurrence and distributional studies. Snow tracking has been used widely in the US (Zielinski and Kucera 1995), Canada, and Scandinavia (Pellikka *et al.* 2005; Hellstedt *et al.* 2006) to monitor populations of diverse carnivores. In open landscapes, snow

tracking even can be conducted from helicopters or planes (Heinemeyer and Copeland 1999).

Carnivore presence can also be determined from hair (from scats, kill sites, or hair snags) using macro- or microscopic examination of hair morphology (Raphael 1994; Teerink 2003). Where all sympatric carnivores and other species with similar hair patterns can be catalogued, hair morphology *may* be diagnostic for species' identification (Oli 1993; Gonzalez-Esteban *et al.* 2006). In many cases, unfortunately, no diagnostic visual or microscopic characteristics exist for species' identification, e.g. black versus grizzly bears (*Ursus Americana vs U. arctos*, Woods *et al.* 1999); and hairs from different parts of the body may have different morphology.

Other sign, such as scrapes, tree nests, latrines, and kills, can also be used to survey for specific carnivores. Identifying sign is a terrific natural history skill, but sign surveys by themselves supply limited information, due to species' misidentification and inability to distinguish individuals. Assuming, however, that species' identity from sign surveys is accurate, "occupancy modeling" (Chapters 2, 11, 16; MacKenzie and Nichols 2004; MacKenzie *et al.* 2006) allows researchers to combine detection/non-detection histories with spatial modeling to estimate and to predict species' occurrence across a landscape. By incorporating estimates of detectability from sign surveys directly, this approach corrects the inherent negative bias present in naïve occupancy estimates (MacKenzie *et al.* 2003; Tyre *et al.* 2003).

4.1.2 Genetic sampling

Noninvasive collection of genetic samples is limited only by the creativity and natural history knowledge of the investigator. Carnivore hairs and scats are the two most commonly collected genetic samples. Hairs are often obtained via snags or rub devices (Figure 4.1). To sample bears, researchers have strung barbed wire around bait, and bears leave hair on the wire when approaching the bait (Woods *et al.* 1999; Mowat and Strobeck 2000; Kendall *et al.* 2009). Sampling bears' natural rub trees can detect bears not sampled by barbed wire corrals (Boulanger *et al.* 2008; Stetz *et al.* 2010). After McDaniel *et al.* (2000) published a protocol for a baited hair-collecting pad, using roofing nails for Canada lynx surveys, this collection device was used to sample Eurasian lynxes (Schmidt and Kowalczyk 2006), ocelots (*Leopardis pardalis*, Weaver *et al.* 2005), and felids in the tropics (Castro-Arellano *et al.* 2008). Rub pads and backtracking putative lynx tracks in snow to collect scats and hairs is more efficient than rub pads alone (McKelvey *et al.* 2006). Zielinski *et al.* (2006) used glue tips to collect hair from small forest carnivores.

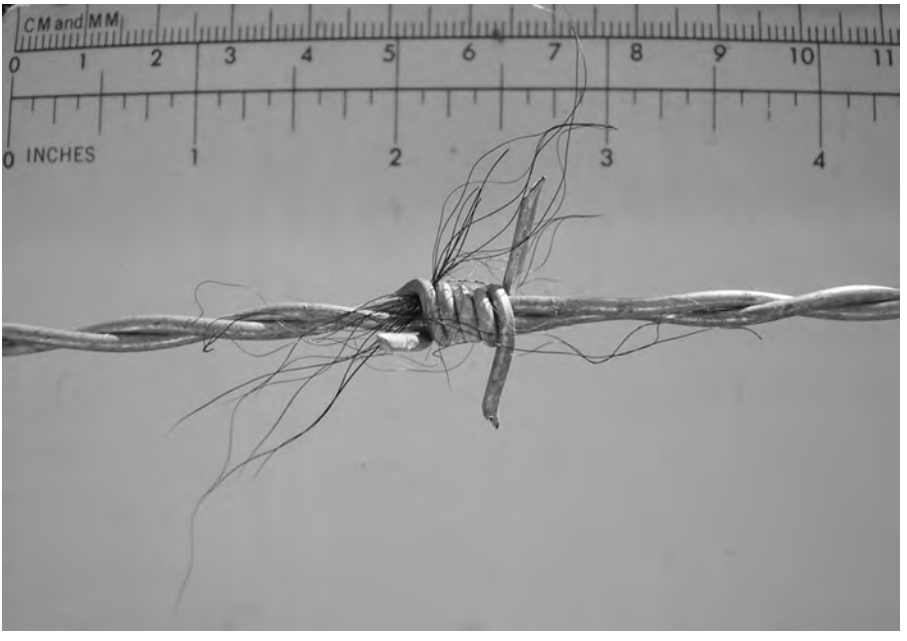

Fig. 4.1 A barbed wire hair snag "capturing" black bear hair for later DNA analysis. As part of a road ecology study, barbed wire was strung along the entirety of an 11-mile stretch of a highway, which was due to be widened. In addition to locating hotspots of road crossing (and identifying areas for potential underpasses), this study examined which sex and individuals were more likely to cross roads and where. Photo courtesy of J. Andrew Trent, Virginia Tech.

Hair-snag devices are usually inexpensive and easy to install but require carnivores to find them (potentially necessitating baits and species-specific attractants) and to rub against them. For some species, the amount of DNA left may be very small (e.g. single hairs or hair fragments). Hairs with follicles provide higher quality DNA extracts than do scats, which have more agents that inhibit and prevent amplification. A single hair, however, usually yields much less DNA than feces. Multiple hairs can usually be pooled to increase DNA yield for species' detection studies, because diagnostic bands for multiple species can be simultaneously visualized. When individual identity is required, however, pooling multiple hairs is risky because it can create false, "new" genotypic individuals (see Alpers *et al.* 2003; Roon *et al.* 2005). Researchers must accept the low DNA yield from single hairs, or perhaps develop a hair snag that allows only one animal to rub it (Beier *et al.* 2005; Bremner-Harrison *et al.* 2006).

Fecal DNA originating from cells sloughed from the intestinal lining and extracted from scat samples can be collected from elusive carnivores, which often deposit scat at prominent sites for intra- and interspecific communication (Gorman and Trowbridge 1989; Barja *et al.* 2005). Typically, scats are collected by walking transects and searching visually. Efficiency can be increased by following animal tracks in the snow, sand, mud, or dust (McKelvey *et al.* 2006; Ulizio *et al.* 2006; Marucco *et al.* 2008).

Researchers also can increase scat-collection rates, even over large, remote areas, by using scent-detecting or scat-detector dogs (*Canis familiaris*; Hurt *et al.* 2000; Wasser *et al.* 2004; Smith *et al.* 2005, 2003; Long *et al.* 2007; MacKay *et al.* 2008). Detector dogs commonly are trained and handled following protocols applied for search-and-rescue dogs (MacKay *et al.* 2008). The dogs must have a strong, object-oriented drive towards a toy or food, which serves as a reward after successful detection. High-performing detection dogs are hard-working, energetic, focused, bold individuals, and are selected independent of breed or sex (Svartberg 2002; Maejima *et al.* 2007; Rooney *et al.* 2007). They require much attention and focused care from trained, professional handlers. A handler needs to learn a dog's behavior and body language to interpret detection alerts correctly under difficult field conditions. Handlers must have knowledge of scent-direction patterns in challenging environments (Shivik 2002; Gazit and Terkel 2003). After a dog alerts its handler (Figure 4.2), the handler investigates the find, being careful about body language so as not to affect the dog's response, and decides if the dog was successful and deserves a reward. Handlers should carry and use target scats during periods of low scat detections to keep dogs motivated and reliable (i.e. prevent false detections to get its toy).

Using scat dogs in a survey design depends on study objectives, habitat, and characteristics of the target species and budget (scat dogs can be expensive). Established trails and roads may be used in some cases, as when DeMatteo *et al.* (2009) surveyed for bush dog scats within 15 m of both sides of trails and roads through thick tropical vegetation. In other cases, opportunistic searches may be made within survey grid cells (Wasser *et al.* 2004; Wultsch 2008; Figure 4.3), or following predefined transect routes (Smith *et al.* 2006; Long *et al.* 2007). If scat dogs follow the trails of individuals of the target species, scats will not be a random or representative sample from the population. This bias is important for some studies. Finally, study design must include tests of scat dogs to document error rate for each individual.

Once collected, samples must be properly stored to inhibit enzymes that degrade DNA. In general, hair samples are easy to store. In dry environments, simply place hairs in individual paper envelopes; in humid climates, dry them quickly and

Fig. 4.2 Example of a sit alert by a scat detection dog, Billy, upon finding a felid scat in Belize, Central America.

completely with silica gel drying agent. For scats, DNA quality can vary depending on collection location, environmental conditions (e.g. UV light, humidity, mold) and the region of the scat sampled (Wultsch 2008). Dry scat samples in silica gel, with at least 5X desiccant per part of sample. Samples can be frozen, but repeated freeze–thaw should be avoided (do not use household freezers with self-defrost). A scraping of a scat to obtain shed epithelial cells can be put into buffer solution in the field, preferably into screw-top tubes to prevent leakage. Liquid storage techniques, such as ethanol (>95%) and DET buffers (at 5–10 parts per part of sample), are excellent for DNA preservation, but ethanol has a tendency to leak.

Other sample types can be stored similarly. For example, DNA collected from saliva with a Q-tip-like swab, either directly from a carnivore or from a prey item bite wound to determine the species and identity of the predator (Sundqvist *et al.* 2008), can be allowed to dry in a paper envelope (avoid plastic bags) or, for longer term storage, placed in Longmire buffer. Urine samples can be collected with swabs or collected directly from snow and kept frozen until DNA extraction (e.g. Hedmark *et al.* 2004; Sastre *et al.* 2009).

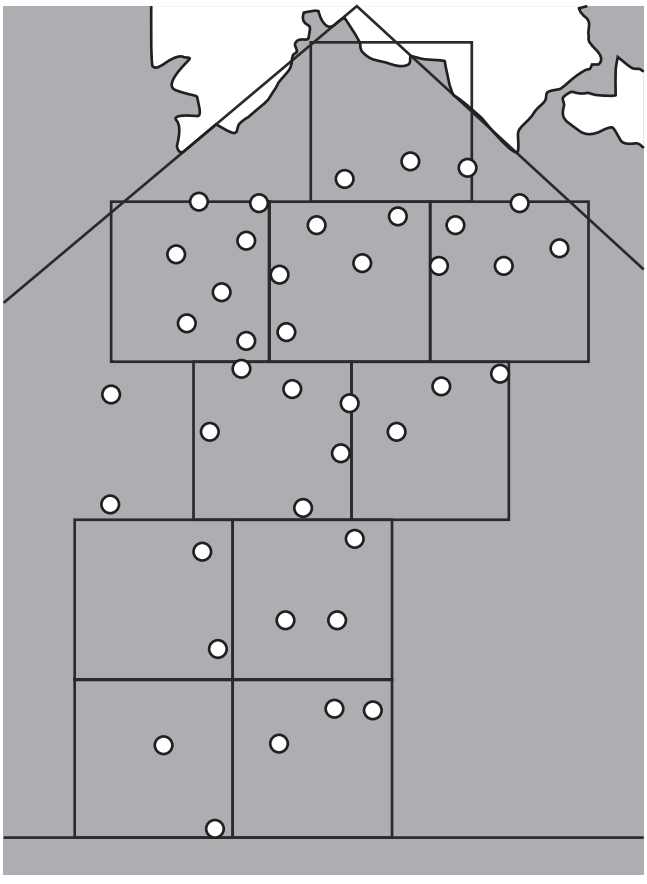

Fig. 4.3 An example of a survey designed specifically for capture–recapture estimates of abundance of felids and to compare two noninvasive survey techniques (remote cameras and molecular scatology) in Belize, Central America. Remote cameras were placed at 1.5–3.0-km intervals for ocelots and jaguars and were operational for 2.5 months. Camera data were collapsed such that every 10 days was one encounter occasion for ~8 encounter occasions for capture–recapture population estimates. A 4 × 4 km grid was superimposed over the camera grid and a scat-dog team searched opportunistically for felid scat for a minimum of 5 km per grid cell. After completing all grid cells in roughly 10 days, the scat-dog team repeated the survey up to five times to create five encounter occasions for mark–recapture. Two camera stations were left out of the analysis due to difficulties in reaching those stations with the scat dog.

Obtaining at least 5–10 known tissue or blood samples—preferably from the same animals for which noninvasive samples were collected—allows optimization of laboratory protocols and determination of genotyping errors (see Section 4.2.1). High-quality samples may include ear tissue punches < or ~5 drops of blood.

Tissue samples can be desiccated in silica, stored in ethanol, or frozen. Blood can be stored dry on filter paper, frozen, or preserved in buffer.

The benefit of silica, ethanol, Longmire or DET buffer preservation is that samples can be stored at room temperature. Nonetheless, freezing samples at –20°C (or colder) is advisable to increase DNA yield. Getting samples to the lab for extraction within a few weeks or months, increases amplification success. Field personnel must be vigilant to guard against cross-contaminating samples during collection. The proper steps include using new latex gloves with each sample, sterilizing instruments with alcohol and flame before each collection, and storing different samples in different, well-labeled containers. Data organization is facilitated through a bar code system using peel-off labels to link physical samples to information (e.g. time, location) on data sheets (Kendall and McKelvey 2008). Methods for extracting and storing DNA are evolving rapidly; readers should see reviews and check the forensic genetics literature (e.g. Oyler-McCance and Leberg 2005; Schwartz and Monfort 2008; Beja-Pereira *et al.* 2009; Morling 2009).

4.1.3 Camera-trap sampling

Photographing wildlife via remotely triggered cameras (camera trapping) emerged in 1877 (Guggisberg 1977) but was little used until the invention of infrared, automatically tripped cameras in the 1980s. Cameras became commercially available, lightweight, and easy to operate. In the mid-1990s, large-scale camera grids were linked with capture–mark–recapture analysis to estimate animal abundance (Karanth 1995; Karanth and Nichols 1998, Chapter 5). The 2000s brought digital camera technology. Widespread, remote camera use has resulted in an increase in carnivore inventories, due to the ability to photograph multiple species at the same site (e.g. Barea-Azcon *et al.* 2007; Datta *et al.* 2008; Tobler *et al.* 2008; Can and Togan 2009; Johnson *et al.* 2009; Pettorelli *et al.* 2009).

Two infrared trigger mechanisms exist in remote camera technology: active and passive infrared systems. An active infrared beam is triggered by an animal breaking an infrared beam that passes from a transmitting unit through the detection zone to a receiving unit. A passive infrared system is triggered by the heat difference between the animal and the environment as the animal moves past a heat and/or motion sensor (Kays and Slauson 2008). While pressure pad and baited string-trip cameras are still useful (King *et al.* 2007), most modern studies use passive infrared systems. Camera flashes at night may cause aversion and may be potentially damaging to the eyes of mammals (Schipper 2007), yet some carnivores appear attracted to a flash, especially large felids (personal experience). Digital camera options now include white flash and infrared flash, but image quality is still low with the latter.

Digital camera durability and reliability are increasing and, most importantly, they do not have the 36-exposure limit of film cameras. Some passive digital

systems can transmit images wirelessly to a base station or laptop computer. Additionally, many models can collect short video sequences.

4.1.4 Endocrine/hormone sampling

Hormones affect physiological processes that maintain homeostasis allowing an animal to cope with its environment. The emerging discipline of "conservation physiology" seeks to understand the physiological responses of animals to environments altered by human disturbance (Wikelski and Cooke 2006). Noninvasive endocrine tools are employed to monitor wildlife populations and individuals (Berger *et al.* 1999; Foley *et al.* 2001; Garnier *et al.* 2002; Sands and Creel 2004; Cockrem 2005). Immunoassays can measure the concentration of select hormones and their metabolites from noninvasively collected samples such as scats (Wasser *et al.* 1988, Creel *et al.* 1997, Barja *et al.* 2008), urine (Thompson and Wrangham 2008, Braun *et al.* 2009), saliva (Queyras and Carosi 2004), and hair (Koren *et al.* 2002). Due to metabolic clearance rates and gut transit time, fecal metabolite concentration represents a cumulative concentration over time (Schwarzenberger 1996). The length of time depends on the species and the stressor, and requires background research in controlled conditions.

Two types of steroid hormones are commonly assessed in noninvasive studies of wildlife endocrinology: adrenal and gonadal. Adrenal hormones, including glucocorticoids (GLCs), also known as stress hormones, are commonly measured as an indicator of overall physiological condition of an individual or a population. Gonadal hormones, such as progestagens, estrogens, and androgens, are used to determine puberty, estrous, ovulation, pregnancy, abortion, and sex (Brown and Wildt 1997; Morato *et al.* 2004; Sanson *et al.* 2005; Graham *et al.* 2006; Dehnhard *et al.* 2008; Herrick *et al.* 2010).

In biological samples, hormones can be assessed through both quantitative and qualitative techniques. Immunoassays and spectrometric techniques can detect small concentrations and immunoassay techniques are used widely in wildlife physiology (Chapter 12). While conservation physiology is an exciting, emerging discipline, multiple cautions exist. Background hormone levels, time-lags in endocrine response, impacts of age, sex, social status, and microflora on metabolite levels, can confound assessments of potential stressors. So far, most studies are correlative and do not directly address cause and effect (Millspaugh and Washburn 2004; Chapter 10). Although chronically elevated GLCs induced by a persistent stressor *can* have negative effects on an organism, including behavioral, reproductive, metabolic, immune, and neurological functions, to

date GLCs have not been linked to meaningful measures of fitness or population dynamics, and are often linked only indirectly to potential stressors.

4.2 Recent tools and advances in noninvasive sampling

4.2.1 Noninvasive DNA techniques

DNA can be collected by sampling hair, scats, urine, regurgitates, saliva, or nearly any other sloughed piece from an animal. The current deluge of noninvasive genetic sampling for carnivores traces its roots to a single development in the late 1980s: the invention and commercialization of the polymerase chain reaction (PCR). PCR "amplifies" DNA, producing millions of copies of the original template DNA, so that researchers can decipher the genetic makeup of organisms from noninvasively collected samples that may be of poor quality or small quantity.

A DNA marker is a sequence of DNA amplified via PCR. Fragment analyses separate targeted pieces of DNA by size. For species' identification, fragments often are amplified from mtDNA because the high copy number increases the probability of amplification for low-quantity, low-quality samples. Often, the size of the amplified DNA itself is not diagnostic for different species, so the amplified product is broken into species-specific pieces, known as RFLPs (restriction fragment length polymporphisms). Different-sized fragments, diagnostic for each species, are produced depending on whether and how mutations have changed the DNA sequences recognized by the endonuclease (Figure 4.4). RFLPs have been applied to differentiate endangered species, such as the San Joaquin kit foxes from other cooccurring canid species (red fox, grey fox, coyote, domestic dog) (Paxinos *et al.* 1997) and to identify species of felids, ursids, and mustelids (Mills *et al.* 2000a; Riddle *et al.* 2003; Vercillo *et al.* 2004; Colli *et al.* 2005; Bidlack *et al.* 2007; Livia *et al.* 2007).

These fragment approaches are fast and inexpensive but they can be limited by potential variation in mtDNA fragment lengths among individuals within a species. In addition, species sampled must be known a priori, and primers and restriction enzymes must have been identified. Where carnivore species are little known, amplified fragments can be sequenced. A nucleotide sequence must then be compared to known sequences archived in a sequence database (e.g. GenBank). Direct sequencing is expensive (though prices are dropping) and can contaminate the signal of the carnivore with that of its prey, if the sample is scat.

A rapidly developing variant of sequenced mtDNA fragments for species' identification is named "The Barcode of Life Initiative" (Savolainen *et al.* 2005; Ratnasingham and Hebert 2007; www.barcodinglife.org). DNA barcoding

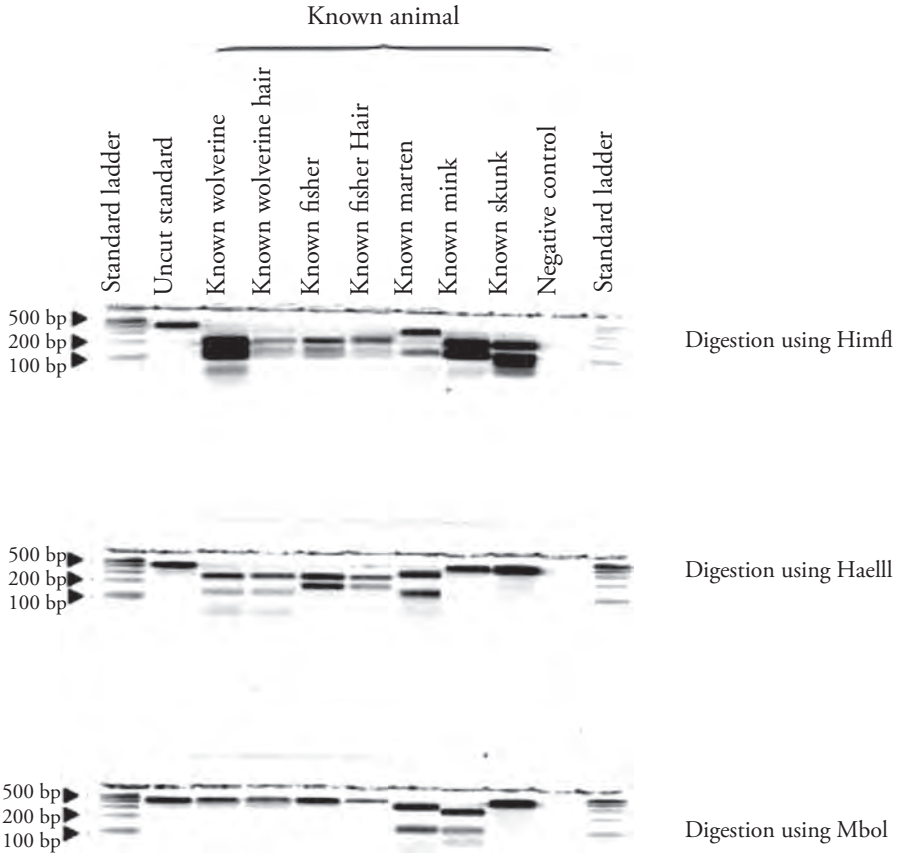

Fig. 4.4 An example of RFLP (restriction fragment length polymporphisms) fragment analysis of mtDNA to distinguish different forest mustelids of the northern USA, using single hairs from noninvasive snags (from Riddle *et al.* 2003). After amplifying the cytochorome *b* region of mtDNA with PCR, the DNA was digested with three different restriction enzymes, creating species-specific fragments that collectively distinguish among different species. The first and last lanes are a molecular ladder that helps to determine size of the bands, and the uncut standard contains a PCR product from a wolverine not subjected to the restriction digests; the negative control is pure water to check for contamination. An example for practice: the first restriction digest (*Hin*fI) distinguishes between marten (with two fragments, of 329 and 113 bp in size) and wolverine (with three fragments of 212, 132, and 98 bp), but wolverine has exactly the same bands as fisher (which appear lighter but are still present). So the next digest (*Hae*III) distinguishes between wolverine (259, 140, and 43 bp) and fisher (259 and 183 bp). Thus multiple restriction enzymes are like multiple morphological characteristics that we might use to tell different species apart.

depends on standardized analyses of a specific DNA region for all species on earth. For animals, the accepted barcode region is a 648-bp region of the mitochondrial Cytochrome c oxidase subunit I (referred to as *cox1* or COI). Barcoding is well-suited to noninvasively collected carnivore samples and has standardized methodology.

Microsatellites, or simple-sequence repeats (SSRs), are short sequences of nuclear DNA repeated between 5 and 100 times, which are widely used for individual-level questions in carnivores. Microsatellite loci typically have high variation within species and are codominant, with alleles displaying Mendelian inheritance. Thus, microsatellites are well-suited to traditional population genetic models and to distinguishing individuals. Sets of highly polymorphic microsatellite loci have been identified for many different carnivore species. Likewise, single nucleotide polymorphisms (SNPs) can be used to address individual-level questions related to genetic variation and population structure, parentage and relatedness, and individual identity (Morin *et al.* 2004; Morin and McCarthy 2007).

The future for carnivore genetic sampling lies in complementing the neutral markers described above with markers that describe, or are linked to, genes of known coding function. These markers, only now being developed for wildlife species, will bring us one step closer to describing fitness attributes directly. These may range from behaviors (e.g. sprint speed), to morphology (e.g. muscle structure), to physiology (e.g. biochemical processing of nutrients).

For carnivore sex determination, a gene present only on the male Y chromosome, such as the SRY gene (testis determining factor), will amplify and be detected in males but not in females. To control for amplification failure, this method usually requires co-amplifying 1 or more microsatellite loci as a control. A second approach amplifies a portion of DNA with alleles of different size residing on both the X and Y chromosomes (Shaw *et al.* 2003). Carnivore sex determination has been applied to fecal samples from sympatric felids in North America (Pilgrim *et al.* 2005) and Asia (Wei *et al.* 2008).

Any time a recorded genotype, or molecular marker, deviates from the actual genotype or marker, a genotyping error has occurred. Although improvements in lab technology and techniques have decreased many forms of genotyping error, it remains an inescapable issue made more prevalent with the low-quantity, low-quality DNA yields of noninvasively collected samples. If unaccounted for, genotyping error could compromise all uses of noninvasively collected DNA samples, including species and individual identity, paternity analysis, occupancy and abundance, gene flow, forensics, and behavior. Some standards for minimizing and measuring genotyping error include following strict protocols, genotyping each specimen multiple times to obtain consensus genotypes (Waits and Paetkau

2005), using statistical metrics to determine levels of genotyping error (McKelvey and Schwartz 2004), and proper use of blind controls (Mills 2002). Once estimated, genotyping error can be incorporated explicitly into parameter estimates (e.g. abundance: Lukacs and Burnham 2005; paternity: Kalinowski *et al.* 2007; Knapp *et al.* 2009).

Genotyping error has been extensively evaluated for abundance estimation, where failing to account for genotyping errors can cause successive captures from the same individual to appear to be from different individuals, biasing the estimates of abundance high (Waits and Leberg 2000). The opposite problem, a low bias, arises when different animals fail to be distinguished due to having too few loci or having too little variation. This phenomenon, termed the "shadow effect" because different animals appear as identical genetic shadows of each other (Mills *et al.* 2000b), decreases as many, highly variable loci become available for most species.

In short, genotyping error is an important consideration in designing and implementing noninvasive genetic studies. It may cost more (e.g. by running each sample multiple times), and it may make the analysis more complicated, but the reward will be more precise and unbiased estimates.

4.2.2 Using noninvasive DNA data

Genetic data can be used to estimate species' distributions. Berry *et al.* (2007) used fecal DNA sampling to provide range information for invasive but cryptic red foxes, a devastating pest, in Tasmania. Canada lynx range distribution on national forest land across the USA was surveyed with mtDNA obtained from hair collected both from baited hair-traps and from backtracking tracks in snow (Mills 2002; McKelvey et al 2006). Nicholson and van Manen (2009) used hair samples to document that site occupancy for black bears decreased after completion of a new highway in North Carolina and that the decrease was not a function of distance from the highway, rather, the highway affected the entire study area.

The ability of noninvasive genetic sampling to identify individuals makes available the entire body of capture–mark–recapture methods for abundance estimation (Chapter 5). Capture–recapture studies use two basic genetic approaches. The first uses multiple discrete "capture" occasions (Otis *et al.* 1978; Huggins 1989). Hair traps, for example, are spread across a landscape in grid-like fashion (Tredick and Vaughan 2009), or opportunistically with regular or clustered spacing (Kendall *et al.* 2009; Robinson *et al.* 2009). Capture periods last several days to weeks, depending on frequency of returning to the traps to collect hair, and data are analyzed with closed capture models (e.g. Program MARK). The second approach uses continuous trapping, wherein individuals can be "captured" multiple times (e.g. hair snared on multiple traps) within a single trapping occasion

(Miller *et al.* 2005; Petit and Valiere 2006). The samples resemble random draws from the population with replacement and can be analyzed with closed capture models in CAPWIRE (Miller *et al.* 2005) or BAYESN (Gazey and Staley 1986; Petit and Valiere 2006).

Noninvasive genetic sampling can extend capture–recapture techniques over time (e.g. years) to obtain multiple estimates of population size. Such data can yield estimates of population growth, survival, and recruitment. In the Alaska range, Prugh *et al.* (2005) used fecal genotyping of 834 scat samples over a 3-year period to estimate coyote survival rates. Interestingly, they found that radio-collared individuals had higher survival rates than uncollared individuals, but that survival did not differ between the sexes. Marucco *et al.* (2008) genotyped 1399 scats from 14 sampling sessions of wolves recolonizing the Western Alps in Italy and France. Using open-population models and AIC model selection they documented that young wolves had lower apparent annual survival than adults, that survival rates were lower in the summer than in the winter, and that population growth over 7 years was positive ($\lambda = 1.04$) but lower than that recorded for other recolonizing wolf populations.

Noninvasive genetic sampling provides researchers with new approaches to use landscape genetics to elucidate conservation challenges. Although the seminal theory for quantifying population structure from genetic data dates back to Sewall Wright (1931), the field of "landscape genetics" has blossomed as a recent integrative discipline to understand how landscape features affect animal movement and local adaptation (Storfer *et al.* 2007; Balkenhol *et al.* 2009; Sork and Waits 2010). F_{ST} (Wright's measure of genetic distance) or coalescent approaches provide relative measures of gene flow, assuming equilibrium between genetic drift increasing divergence and gene flow decreasing it. Because this equilibrium would have been achieved many generations previously, before recent human-caused population fragmentation, these measures may essentially provide a window into historic levels of connectivity. By contrast, current levels of gene flow (interpopulation movement followed by breeding), analogous to immigration-and-reproduction events measured by radio-telemetry, can be estimated by genetic assignment tests. As an example, Proctor *et al.* (2005) used hair traps to survey for grizzly bears on both sides of a major highway just north of the US–Canada border. They used two approaches to test for migrants. First they used area-specific allele frequencies in a likelihood-based assignment test (Paetkau *et al.* 1995). Individual were "assigned" to the area with the highest probability of occurrence. Second, they used a model-based clustering method in program STRUCTURE (Pritchard *et al.* 2000), which clusters individuals into groups through iterative assignments and probabilities of origin. Individuals that are repeatedly assigned to a group other than where they

were captured, are considered putative migrants. The results were striking: a surprisingly small amount of migration that was heavily sex-biased occurred (mostly males and only one female), suggesting that demographic connection had been severed across their entire range in southern Canada by the highway and associated settlements.

It is now axiomatic that maintaining genetic variation is important, both to maintain long-term evolutionary potential in a changing environment and to minimize the short-term demographic effects of inbreeding depression (Frankham 2005; Mills 2007; Chapter 9). Noninvasive genetic samples can provide estimates of genetic variation (e.g. heterozygosity and polymorphism) and effective population size (Tallmon *et al.* 2008). More importantly, sampling over time, or in fragmented vs. control sites, can document potential *decreases* in heterozygosity, which can translate into decreases in survival or reproductive rates via inbreeding depression. Inbreeding depression, in turn, can decrease population growth rate and decrease population viability (Mills and Smouse 1994).

Noninvasive genetic sampling has also become important for assessing the taxonomic status of individuals. For example, the primary threat to the persistence of reintroduced, endangered red wolves (*Canis rufus*), is hybridization with coyotes; a microsatellite nDNA test of wild-born pups (Adams and Waits 2007) allows managers to detect and remove hybrids before they can interbreed with the extant red wolves. Additional insights into hybridization can be revealed using mtDNA, whose maternal inheritance indicates the direction of hybridization. Because coyote mtDNA is found in gray wolves but not vice versa, hybridization between coyotes and wolves occurs by way of male wolves mating with female coyotes (Lehman *et al.* 1991).

Of course, the fact that genetic sampling can provide diagnostic identification of both species and individuals has immediate and broad implications for forensics and solving wildlife crimes involving carnivores. Millions and Swanson (2006) hypothesized that bobcats in Michigan were being poached from the Lower Peninsula (LP) but registered by hunters as harvested from the Upper Peninsula (UP), where bag limits were higher. Microsatellites markers and assignment tests documented that some bobcats claimed as harvested in the UP were genetically assigned to the LP. In a more condemning example, Caniglia *et al.* (2009) extracted DNA from wolf canine teeth on a necklace to show that the teeth belonged to six individual Italian wolves (a legally protected species), including a male and a female wolf recently found dead.

Genotyping individuals within a population allows researchers to calculate relatedness and thereby to examine social and mating structure. Gotelli *et al.* (2007) used fecal DNA analysis to determine paternity of cheetah (*Acinonyx*

jubatus) cubs and found that adult females were surprisingly promiscuous. Not only did 43% of litters have multiple fathers, females mated with unrelated males within an estrus cycle and mated with different males in subsequent breeding seasons.

4.2.3 Data collection, handling, and analyses with remote cameras

Type of cameras used, placement, and duration of camera-trapping studies depend on the goals of the study (Figure 4.3), land cover, and budget. Deploying large numbers of remote cameras is expensive. Study design, deployment, site selection, equipment management, minimizing theft and wildlife damage, camera expense, reliability, and sensitivity have been reviewed by Swann *et al.* (2004), Kays and Slauson (2008), and Long and Zielinski (2008). Several websites compare camera performance and prices. Regardless of study goals, one must plan for the substantial data-management required in any camera-trapping study. Sifting through photographs (either film or digital) and entering them into a useful database often takes more time than deploying and monitoring cameras in the field. While a study may target only one carnivore species, entering all data on all non-target species, including humans, is important, as this information can become useful for determining potential competitors, distribution of prey, linking trapping rates (photos taken) of the target carnivores to trapping rates of prey, and human use of the study site (Figure 4.5).

No standard for number of camera-trapping stations, spacing between cameras, or duration of surveys exists for documenting carnivore presence or conducting species' inventories (Kelly 2008). Camera placement and spacing is flexible and often includes targeting likely areas with more cameras, while not surveying unlikely areas. Many studies use a minimum of ~1000 trap nights per study site, but variable objectives and detectability of the target species affects trap nights needed. The lower the detectability of target carnivores, the more trap nights are needed. Increasing camera saturation can decrease the total number of trap nights needed to detect target carnivores (Wegge *et al.* 2004).

Camera trapping was first used in conjunction with capture–recapture models to estimate abundance and density for tigers (Karanth 1995; Karanth and Nichols 1998) and then modified for other boldly marked felids (e.g. Silver *et al* 2004; Maffei *et al.* 2005; Di Bitetti *et al.* 2006; Dillon and Kelly 2007) and carnivores with ear tags (Thompson 2007) or uniquely identifiable ear streamers (Bridges *et al.* 2004a). The technique can even be used for subtly marked species, such as pumas (Kelly *et al.* 2008) and red foxes (Sarmento *et al.* 2009), albeit with more constraints and lower confidence. Alternatively, Rowcliffe *et al.* (2008) treated

Fig. 4.5 Photographs from remote cameras: (a) R. Felix Jean and A. Vonjy Arindrano (WCS/Madagascar) conducting a camera check and demonstrating double documentation of date, camera number, and station number on placard and time embedded on digital image. Information is also recorded on a data sheet and input into a computer database. (b) Non-target species, such as tamanduas (Mountain Pine Ridge, Belize), are often caught on remote cameras and can reveal interesting behaviors. Non-target species (even humans) can prove valuable for biodiversity surveys and potentially can be linked to presence or trapping rates of target species. (c) Bears are notorious for damaging remote cameras, as in this photograph taken near Mountain Lake Biological Station, Virginia. Every remote camera study should plan for sufficient cameras to replace those that are stolen, vandalized, damaged, or malfunction. (d) Remote film camera captures two jaguars in Hill Bank, Belize demonstrating differences in coat patterns that make individual identification possible for mark–recapture studies.

contact rates between cameras and animals using an "ideal gas" model to scale trapping rate linearly with density.

Survey design for estimating abundance using remote cameras is an active area of research (Chapter 5). Most studies use a fixed grid with a minimum of 20 stations with 2 cameras per station, at a spacing that ensures that each individual has a reasonable probability of capture. Capture histories are constructed for individual animals photographed at each site and data analyzed with closed capture models. Because camera grids are often different sizes and can change shape in longitudinal studies, abundance must be converted to density to make comparisons.

Unfortunately, estimating the effective trap area is a sticky problem. One can calculate half the mean maximum distance moved (MMDM) between camera locations among all individuals re-photographed at least once (Karanth and Nichols 1998), and apply this as a buffer around the trapping grid. Wilson and Anderson (1985) provide an entry to the extensive literature on calculating densities by MMDM approaches. New analytical approaches that estimate density directly through spatially explicit capture–recapture models avoid the potential pitfalls of the *ad hoc* mean maximum distance-moved approaches (e.g. Efford 2004; Gardner *et al.* 2009). Comparative analyses suggest that MMDM models can substantially overestimate density compared to spatially explicit capture–recapture models (Obbard *et al.* 2010; Gerber *et al.* 2011).

For long-term, longitudinal camera-trap studies on naturally marked individuals, the Holy Grail is to estimate survival and recruitment. To date, few studies have reached this goal. Karanth *et al.* (2006) used 9 years of data from remote cameras on 74 individual tigers to estimate abundance, population growth rate, survival, recruitment, temporary immigration, and transience using Pollock's "robust-design" (Pollock 1982; Pollock *et al.* 1990; Chapter 5).

The potential to use remote cameras for large-scale carnivore distribution studies is tremendous. Indeed, many countries are required to monitor biodiversity under directives such as the Convention on Biological Diversity (Mace and Baillie 2007). Pettorelli *et al.* (2009) combined camera-trap surveys across 11 sites in Tanzania, East Africa, with ecological niche factor analysis (ENFA; Chapter 10) to reveal distributional and habitat use patterns for 23 carnivore species. ENFA techniques (Hirzel *et al.* 2002) use presence-only data to determine habitat features that promote species' presence. An advancement from the ENFA approach is the occupancy-based approach, which also reveals habitat-use patterns and predicts carnivore occurrence across a landscape, by explicitly modeling detectability as a function of species and environmental variables (MacKenzie 2005; Chapter 10). Thorn *et al.* (2009) used baited camera-traps to estimate brown hyaena (*Hyaena brunnea*) occupancy in South Africa and Linkie *et al.* (2007) combined remote cameras and occupancy to gain information on sun bears (*Helarctos malayanus*).

Many studies have used remote cameras to assess finer scale habitat-use patterns, including use of existing trails by carnivores (Dillon and Kelly 2007; Harmsen *et al.* 2010; Davis *et al.* 2011). Trail systems funnel carnivores past cameras, facilitating photo-captures but potentially biased estimates of density. Alternatively, a researcher can establish a trail system for camera trapping in trailess areas, as carnivores are likely to begin using these paths (Maffei *et al.* 2004). Some carnivores, such as coyotes, however, may be wary of baited cameras on trails (Sequin *et al.* 2003). Other studies have used geographical information systems to extract

land-cover data from circular buffers surrounding camera traps (Kelly and Holub 2008; Davis *et al.* 2011).

While limited in scope, camera traps can give insight into carnivore behavior, particularly for describing activity patterns for members of a single species (Bridges *et al.* 2004b; Vanak and Gompper 2007) or of sympatric carnivores studied simultaneously to gain insight into coexistence (Grassman *et al.* 2006a; Chen *et al.* 2009; Di Bitetti *et al.* 2009; Harmsen *et al.* 2009; Lucherini *et al.* 2009). Stevens and Serfass (2008) used remote cameras to examine group composition, seasonality, and activity patterns of river otters at latrines. Hunter (2009) used remote *video* cameras to record the responses of predators to taxidermy models of striped skunks and gray foxes, and learned that carnivores use both coloration and body shape to recognize and to avoid noxious species. Bolton *et al.* (2007) used a digital infrared camera system to monitor predation events at the nests of ground-nesting lapwings (*Vanellus vanellus*) and tree-nesting spotted flycatchers (*Muscicapa striata*). Finally, remote cameras are used extensively to identify species and use rates of highway crossing structures, such as under- and overpasses (Clevenger and Waltho 2000).

4.2.4 Data collection, handling, and analyses for endocrine studies

Techniques that measure steroid metabolites excreted in urine or feces provide an avenue for noninvasive research on the physiology of free-ranging carnivores. Urine is often impractical to procure from wild, free-ranging carnivores but could be feasible for endangered species in *ex situ* conservation programs. Steroids in urine are a reliable indicator of ovulation in carnivores (Dehnhard *et al.* 2006; Durrant *et al.* 2006).

Metabolic studies in captive carnivores show that adrenal and gonadal steroid metabolites are excreted at measureable concentrations predominantly in feces (Brown and Wildt 1997; Young *et al.* 2004). Scats should be handled with gloves and stored individually in resealable plastic bags or polypropylene tubes. The scats should be homogenized prior to lab analysis to ensure representative hormonal concentrations in the sample, since hormones and metabolites may be unevenly distributed. Moreover, after defecation, microflora present in a scat can produce enzymes that further metabolize steroids, altering concentrations. Freezing is recommended to arrest microbial and enzymatic activity, even if the samples have been dried or hormones extracted, unless metabolite stability is confirmed beforehand (Möstl and Palme 2002; Lynch *et al.* 2003; Millspaugh and Washburn 2004). Freeze/thaw events should be avoided. Degradation of steroid hormones in feces can be caused by ultraviolet light, humidity, and temperature. Additionally, degradation rates are influenced by time, diet, and species-specific intrinsic

intestinal flora (Touma and Palme 2005; Schwartz and Monfort 2008). These factors may cause fecal glucocorticoid metabolites (FGMs) to increase through time (Washburn and Millspaugh 2002) or decrease (Pelican *et al.* 2007). To address the possible effects of environmental exposure on scats over time, a pilot degradation study under expected environmental conditions is essential *prior* to field collection, especially because usually there is no reliable way to age scats in the field. For example, a recent study on captive jaguars found that FGMs remained relatively constant for 4 days after defecation giving researchers a 4-day cyclical rotation for collecting scat samples in the field (Mesa, Kelly, Brown, unpublished data).

Because metabolism of steroid hormones differs for each species, laboratory procedures should be validated for the target species *prior* to a field study. Both type and concentration of metabolites will be unique for each species and type of biological sample. Validation involves three components. First, endocrine physiology can be evaluated either by stimulating endocrine organs (e.g. adenocorticotropic hormone, ACTH challenge) or by monitoring physiological events (e.g. acclimatization, recovering from surgery, estral cycles, etc). Subsequently, a metabolite analysis using high-performance liquid chromatography (HPLC) or mass spectrometry facilitates the selection of candidate immunoassays for validation and provides information about metabolism and gut transit time. The use of captive individuals under controlled settings is strongly recommended. Second, hormonal extraction procedures are required to solubilize steroid hormones present in feces, usually through agitation or heating extraction. Hand agitation is practical for field conditions. Heating extraction ("boiling") is the best method of extraction and is often used to corroborate agitation methods. Third, immunoassay selection is based on both affinity of the antibody to the desirable metabolite (obtained from HPLC analysis) and cross-reactivity of the antibody with other hormonal metabolites (Millspaugh and Washburn 2004; Young *et al.* 2004; Palme 2005; Touma and Palme 2005; Keay 2006).

A stress response can be classified as either acute or chronic, depending on the length of exposure, intensity of the stressor (i.e. threat), and the ability of the individual to find a physiological balance (i.e. acclimatization or acclimation). The acute stress response is highly conserved phylogenetically across vertebrate taxa (Romero 2004); thus the mechanism is considered adaptive (Boonstra 2005; Nelson 2005). In fact, acute stress allows an organism to modulate its metabolism, redirecting resources from innate processes, like digestion, growth, immune function, and reproduction, to counter an immediate threat (Nelson 2005). When a stress response is sustained over extended periods of time, the organism is thought to be experiencing chronic stress, also known as distress.

Moberg (1985) noted that the neuroendocrine response to stress has the greatest potential to indicate the impact of stress on an animal's overall well-being. Wildlife endocrinology has demonstrated correlations between physiological responses of individuals to anthropogenic disturbances in ecosystems although connections to fitness or population dynamics are limited. For example, Barja *et al.* (2007) showed a direct correlation between unregulated tourism and the level of FGMs in European pine martens. FGMs in elk (*Cervus canadensis*) and wolves correlate with the intensity of winter snowmobile activity in Yellowstone National Park (Creel *et al.* 2002), though this response had little to no negative effect on the population dynamics of wolves. In a more complex example, dominant female meerkats (*Suricata suricata*) during pregnancy employ stressful evictions to suppress reproduction among subordinates (Young *et al.* 2006, 2008). Subordinates have higher concentrations of FGMs and reproductive down-regulation via decreased conception rates and increased abortions. Dominant females benefit by diminishing competition for limited care among their own and subordinate litters, and through lowering the chances of infanticide by the subordinate females (Young *et al.* 2006).

Reproductive status in wild captive animals has been monitored for decades through noninvasive endocrine sampling (Schwarzenberger 2007). Sex determination also can be achieved using fecal gonadal steroids (Barja *et al.* 2008).

Noninvasive endocrine monitoring has management implications for carnivore translocations, reintroductions, and rehabilitation. While no studies currently exist for carnivores, noninvasive fecal endocrine monitoring during translocation and after release can document time required to return to pre-translocation FGMs (Franceschini *et al.* 2008).

4.3 Combining noninvasive and traditional approaches

4.3.1 Comparative approaches among noninvasive techniques

Comparative studies have helped to increase efficiency and to identify which noninvasive techniques work best for a particular species. Long *et al.* (2008a: tables 12.1 and 12.2) provided a list of survey methods and their attributes for North American carnivores.

Several comparative studies designed specifically to assess noninvasive techniques for carnivores are instructive. Harrison (2006) found that a scat dog produced 10 times the number of bobcat detections as did remote cameras, hair-snares, and scent-stations, but the dog was the most expensive and time-intensive technique. This study did not, however, compare methods for determining the number of individual bobcats identified, which could be achieved through remote camera

identification and DNA analysis of feces and hair. Long *et al.* (2007) used a scat-detector dog, hair snares, and remote cameras to survey carnivores in the northeast USA. All three techniques detected black bears but hair snares did not detect fishers or bobcats. They also found the scat-detector dog technique to be the most expensive but it yielded the highest detection rate, rendering it the most cost-effective in the long run. Gompper *et al.* (2006) compared track plates, remote cameras, snowtracking, and scat surveys and found that no one particular technique was best for all species within their carnivore guild. Track plates detected more small carnivores, such as martens and weasels (*Mustela* spp.), and were equivalent to camera traps for midsized carnivores, such as raccoons, fishers, opossums, and domestic cats. Cameras were efficient for bears, while scat surveys and snowtracking were the best methods for coyotes.

Using remote cameras, sign (scat, scrapes, tracks, scent marks) and molecular scatology McCarthy *et al.* (2008) found that low capture and recapture rates of snow leopards (*Uncia uncia*) with remote cameras caused capture–recapture estimates of abundance to be unreliable. Molecular scatology held promise, however, and sign surveys could be most efficient once corrected for observer bias and environmental variance. Tiger (*Panthera tigris*) abundance estimated from genetic capture–recapture models closely matched that from camera traps (Mondol *et al.* 2009).

In short, the choice of noninvasive techniques must be tailored to the target species and study objectives.

4.3.2 Combining traditional with noninvasive approaches

Traditional and noninvasive methods have been compared occasionally using carnivores. Using scat surveys, scent-stations, baited camera-trapping, and live trapping in baited box traps to estimate carnivore species' richness in the Mediterranean, Barea-Azcon *et al.* (2007) found that scent-stations and scat surveys were most efficient logistically and economically over a large spatial scale. They detected genets, however, only with scent-stations and baited cameras and box traps were best for wildcats.

Several studies have simultaneously used radio-telemetry and camera trapping to estimate carnivore densities. Two showed that camera trapping can grossly overestimate carnivore densities (Soisalo and Cavalcanti 2006; Dillon and Kelly 2008), while another found high congruence of the two methods (Maffei and Noss 2008). Balme *et al.* (2009a) used track counts and remote cameras to estimate the size of a known population of radio-collared leopards (*Panthera pardus*) in South Africa. The most accurate estimate of the known population came from camera trapping data, when it was supplemented by movement data from radio-telemetry.

Traditional camera-trapping methodology, however, did not result in gross overestimates, and track counts provided some reliable results.

4.3.3 Data quality and integrity in noninvasive surveys

Noninvasive techniques open doors to sampling carnivores in ways never imagined 30 years ago. The ease of sampling provides an opportunity for involving masses of untrained volunteers, with the potential for creating an unprecedented volume of carnivore data from the field. This strength of noninvasive sampling is also a weakness, as it creates novel problems in maintaining data quality and integrity. Fundamentally, this means that extra steps must be taken in the field to ensure high data quality. For example, training of volunteers and availability of detailed protocols, are essential in any large-scale noninvasive survey.

For camera surveys, an additional necessary step includes double, or triple documentation of camera station locations, dates, and researchers present (Figure 4.5a). Trigger each remote camera in a survey during camera setup, and also during each camera checking, with a placard that reads the station location and date (at minimum), even if this information is already embedded in memory card (Figure 4.5).

Similarly, noninvasive genetic studies require extra checks on data quality, but can still suffer if field collectors fail to follow protocols. The National Lynx Survey, a 3-year noninvasive study to determine lynx distribution across 16 states, provides an instructive example (Mills 2002, 2007). Several hundred personnel initiated the placement and checking of more than 21 000 hair rub pads, following detailed protocols provided by the principal investigators. The study was a success in the ambitious scope of sampling across the species' range in the USA, in that 80% of hair samples collected could be identified to species. Nevertheless, a few personnel threatened the integrity of the entire study by mislabeling samples (Thomas and Pletscher 2002). Fortunately, the study had in place essential checks at both the field and lab level, including the critical design feature that hair collection was only the first step in evaluating lynx presence; follow-up snowtracking and trapping efforts were built into the study to separate actual lynx populations from fur-farm escapees, transient individuals, or mislabeled samples.

Noninvasive approaches draw from cutting-edge advances in molecular genetics, biostatistics, population biology, endocrinology, and epidemiology. Carnivore ecologists must learn more scientific disciplines, in greater depth, than ever before for appropriate application of noninvasive sampling. Though daunting in complexity, the promise of greater understanding of carnivores via noninvasive sampling should be seen as a rallying call across disciplines. Never has there been a more challenging, but also exciting and productive time to study carnivores.