Chapter 9

Genetic and Endocrine Tools for Carnivore Surveys

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Modern literature and Hollywood proved decades ahead of science in imagining the information that could be obtained from single hairs or feces. Indeed, from Aldous Huxley's *Brave New World* (1932) to the cult movie *GATTACA* (Columbia Pictures Corporation 1997), writers and producers foreshadowed the scientific value of noninvasive samples. In the 1990s, with the advance of both molecular genetics and endocrine biology, forensic scientists developed tools to determine the identity, sex, health, and social status of humans from samples left at crime scenes (e.g., hair, scat, urine, saliva). As with many technological advances in human biology, these developments soon transferred to other disciplines—including wildlife biology.

In this chapter, we review the state-of-the-art tools that molecular and endocrine biologists employ to learn about carnivores and other wildlife through noninvasive means. The chapter is divided into three sections, with the first describing advances in molecular ecology, the second recounting advances in endocrinology, and the final section briefly discussing the synergy obtained by combining DNA and endocrine tools for understanding carnivore ecology. The primary objectives of this chapter are to (1) provide a general overview of laboratory methods and demonstrate their application via examples; and (2) share practical information with field biologists regarding what and how to sample, and how to treat samples in the field to optimize efficacy in the molecular genetics or endocrinology laboratory.

We don't expect readers to be experts in genetics or endocrinology after reading this chapter, yet we believe that a limited understanding of laboratory methods is helpful—if only to aid in communication with laboratory scientists. Thus, rather than exhaustively describe all existing laboratory techniques, we include material on commonly asked questions and how these questions are typically addressed in the laboratory. Our goal is not to provide a lab manual but to create a useful resource for field biologists. We have strived to balance simplification with precision and to be neither pedantic nor so technically thorough that we fail to convey our meaning. We have also included a glossary of genetic and endocrine terms (appendix 9.1) to assist readers in need of more information and have emphasized glossary terms upon first use in the chapter. Last, we have chosen to combine genetics and endocrinology into one chapter because there are many cases where a researcher may wish to obtain both endocrine and genetic information from the same sample. In some ideal situations, the information obtained from these disciplines is complementary. The last section

of this chapter therefore attempts to integrate genetics and endocrinology.

Genetic Approaches for Studying and Monitoring Carnivores

Our understanding of the natural world has been dramatically expanded by the field of molecular biology. Yet, modern breakthroughs in technology, the hype of this technology in popular culture, and the remarkable applications of new tools for answering age-old questions have lead to some confusion about the realistic abilities of molecular genetic techniques in the context of wildlife research. While it is true that noninvasive genetic sampling, coupled with molecular biology tools, has proven to be very effective at answering important management, evolutionary, and ecological questions (table 9.1), these tools are not a panacea. Here we try to separate fact from fiction in terms of what can be accomplished with noninvasive genetic sampling.

A Primer on Molecular Genetic Tools for Studying Wildlife

When used to best effect, molecular data are integrated with information from ecology, observational natural history, ethology, comparative morphology, physiology, historical geology, paleontology, systematics, and

Table 9.1. Examples of the use of genetic sampling to address objectives pertinent to carnivore ecology, management, and conservation

Objective	*Species*	*DNA source material*	*Reference*
Abundance	Brown bear	Feces	Bellemain et al. 2005
	Eurasian badger (*Meles meles*)	Hair	Frantz et al. 2004
Relative abundance	Coyote	Feces	Kohn et al. 1999
	Mountain lion	Hair	Ernest et al. 2000
Occupancy	Fisher	Hair	Zielinski et al. 2006
	Eurasian lynx (*Lynx pardinus*)	Feces	Palomares et al. 2002
Trend in abundance	Brown bear	Hair	Boulanger et al. 2004a
	Brush-tailed rock wallaby (*Petrogale penicillata*)	Feces	Piggott et al. 2006
Hybridization	Red wolf	Feces	Adams et al. 2003
	Canada lynx	Hair	Schwartz et al. 2004
Paternity and relatedness	Wombat (*Vombatus ursinus*)	Hair	Banks et al. 2002
	Gray wolf	Feces	Lucchini et al. 2002
Sex identification	Ursids	Feces	Taberlet et al. 1997
	Felids	Hair	Pilgrim et al. 2005
Diet assessment	Felids	Feces	Farrell et al. 2000
Sex specific movement	Brown bear	Hair	Proctor et al. 2004
	Wolverine	Feces	Flagstad et al. 2004
Turnover rates and survival	Coyote	Feces	Prugh et al. 2005
	Wolverine	Hair	Squires et al. 2007
Phylogeography and population genetics	Dhole (*Cuon alpinus*)	Feces	Iyengar et al. 2005
	Louisiana black bear (*Ursus americanus luteolus*)	Hair	Triant et al. 2004
Spatial organization	Eurasian otter (*Lutra lutra*)	Feces	Hung et al. 2004
Landscape genetics	American black bear	Hair	Cushman et al. 2006
	Eurasian otter	Feces	Hobbs et al. 2006

> other time-honored disciplines. Each of these traditional areas of science has been enriched, if not rejuvenated by contact with the field of molecular genetics. (John Avise 2004)

The majority of noninvasive genetics studies have used DNA as a diagnostic **marker** to acquire information about difficult-to-study species. For instance, we can determine species identification, sex, and individual identification from a hair sample using diagnostic molecular genetic tools. In this context, noninvasive genetic sampling has been used to address questions of occupancy, abundance, and geographic range (see table 9.1), and when these metrics are collected over time, for genetic monitoring purposes (Schwartz et al. 2007). Bellemain et al. (2005), for example, collected brown bear (*Ursus arctos*) feces throughout south-central Sweden over two consecutive years. Using individual identification information from diagnostic DNA markers, along with four approaches to estimating abundance (two rarefaction indices, a Lincoln-Peterson estimate, and a closed capture model in program MARK), the authors were able to arrive at estimates of abundance for each sex per year.

Noninvasive genetic samples can also be used in a population genetic framework. Population genetics is the study of the distribution and frequency of genes. In this context, noninvasive genetic sampling has been used to investigate effective population size, gene flow, mating systems, genetic diversity, and relationships between populations of many species (Schwartz et al. 1998; Manel et al. 2003; Miller et al. 2003; Wisely et al. 2004; Schwartz et al. 2004; Leonard et al. 2005). Specifically, noninvasive genetic sampling has provided new means for collecting population genetic samples from species that are otherwise difficult to study. Cushman et al. (2006), for instance, used DNA from noninvasive hair snares, coupled with population and landscape genetic analyses, to determine the effects of roads, forest cover, slope, and elevation on black bear (*Ursus americanus*) movement.

Contemporary biologists may take for granted the ability to obtain either diagnostic or population genetic information from genetic samples, but it wasn't until Sir Alec Jeffreys began studying DNA variation and the evolution of gene families through the use of "hypervariable" regions of human DNA that molecular biologists were able to produce a **genetic fingerprint** (Jeffreys 1985a, b; see Avise 2004 for a review of earlier isozyme research). Jeffreys discovered that particular regions of the human genome, which consist of short sequences repeated multiple times, also contain a "core sequence" that could be developed into a tool called a **probe**. Further, Jeffreys recognized that these probes could be used to explore multiple regions of the human genome that also contain tandem repeats (two or more nucleotides sequentially repeated (e.g., CATG, CATG, CATG)—ultimately producing something biologically akin to a barcode—such that each individual has a unique genetic signature.

This technique became known as DNA fingerprinting, and instantly became a staple of forensic and paternity work worldwide. Two years after the development of DNA fingerprinting, Wetton et al. (1987) found these same Jeffreys probes to be useful for studying house sparrows (*Passer domesticus*) in the United Kingdom. Within a decade, DNA fingerprinting was common in many wildlife studies, rewriting conventional wisdom on mating systems and gene flow among populations (Wildt et al. 1987; Lynch 1988; Burke et al. 1989).

Initially, there were two major impediments to DNA fingerprinting for addressing wildlife issues. First, although Jeffreys probes provided a DNA "barcode," it was typically not possible to know which bars were associated with which **locus**. Thus, population genetic models that relied on locus-specific information needed to be adapted or abandoned. Second, high quantities of high-quality DNA were required for these barcodes to be visible on a standard electrophoresis gel. DNA fingerprinting from hair and other samples containing minimal or degraded DNA was therefore unreliable or impossible.

The next two breakthroughs, which have led to the recent boom in genetic techniques, were the development of the **polymerase chain reaction** (**PCR** and the discovery of new classes of genetic markers (Saiki et al. 1988; Tautz 1989; Weber and May 1989). PCR is the "amplification" of a gene, part of a gene, or part of any section of the genome. The process can be likened to a molecular photocopy machine, where a few short DNA fragments are copied many times, ultimately allowing visualization of the PCR product on a standard electrophoresis gel. PCR is conducted in a thermal cycler, which heats and subsequently cools a chemical process to precise temperatures during multiple steps (figure 9.1). Originally, during the PCR process, a critical enzyme (called a polymerase) would break down when DNA strands were heated, making PCR extremely labor intensive, as more polymerase needed to be added during every cycle in the process. The process was greatly facilitated by the discovery of a thermostable enzyme called ***Taq* polymerase** (derived from the organism *Thermus aquaticus*, discovered in hot springs in Yellowstone National Park, USA), which allows the polymerase chain reaction to be subject to extreme temperature increases and decreases without disintegrating the polymerase.

Primers are critical components of the PCR reaction. A forward and a reverse primer together act as

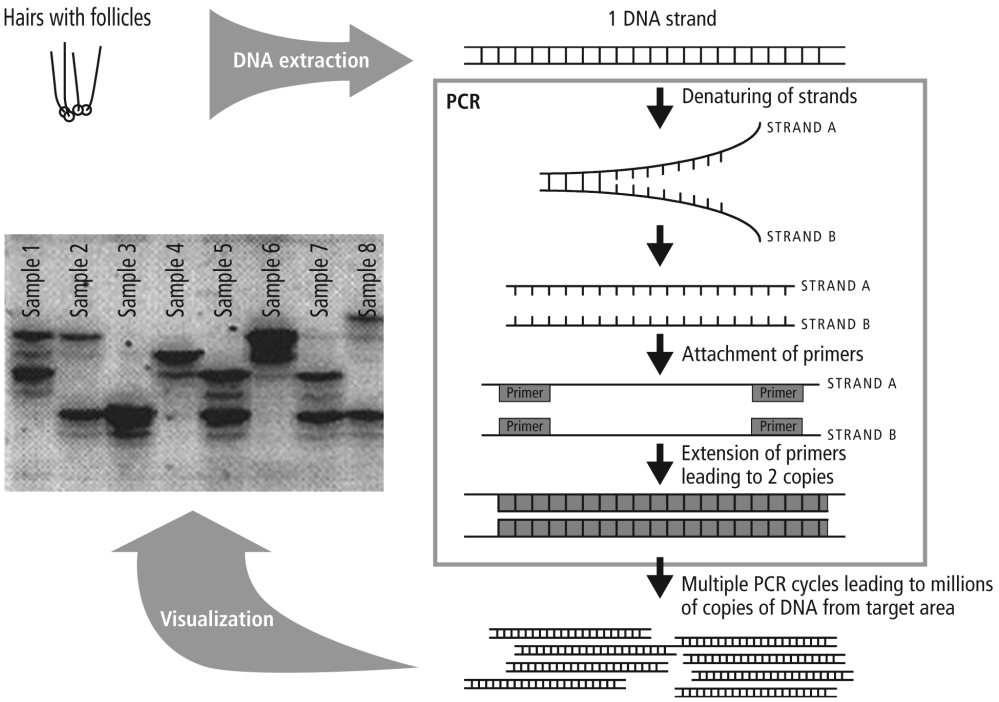

Figure 9.1. Schematic illustrating the process of deriving individual identification from hair samples. This process begins with DNA extraction, which produces DNA strands. Next, a particular region of the DNA is amplified using (in this case) microsatellite primers, and the polymerase chain reaction (PCR). The PCR process involves three major steps: (1) the denaturing of the double-stranded DNA molecule; (2) the attachment of primers at a particular locus in the genome; and (3) the extension of these primers to produce a copy of the original locus. After mulitple PCR cycles, millions—if not billions—of copies of the region are created, and can then be visualized on an electrophoresis gel. This gel image shows eight Canada lynx samples evaluated at one microsatellite locus. Even with one locus, multiple individuals can already be discerned, but samples 5 and 7 produce the same banding pattern at this locus. Ultimately, when additional loci were run, these two samples were determined to be from different individuals.

bookends denoting the section of the genome to be copied. These primers can originate from either the mitochondrial or nuclear genomes of an organism. Deciding whether to examine sections of the nuclear or mitochondrial genome will depend largely on the goals of the study. For instance, if the goal is to determine species from hair or fecal samples, the mitochondrial genome is typically used. **Mitochondrial DNA** (mtDNA) is often less variable within a species than **nuclear DNA** but variable between species (see *Wildlife Genetics in Practice: A Hypothetical Example* later in this chapter). If the goal is to produce individual identification or fine-scaled population genetic information, however, nuclear DNA is often preferred.

Currently, **microsatellites** are one of the most common genetic tools for producing individual identification from noninvasive genetic samples and for conducting population genetic analyses. Microsatellites belong to a class of primers that contain variable numbers of tandem repeats—in general, these repeats are two to five base pairs in length (figure 9.1). They are highly variable in nearly all vertebrates, which ultimately allows the differentiation of individuals within a population.

Microsatellites have several advantages over Jeffreys DNA fingerprinting probes. First, microsatellite **loci** are codominant markers, meaning that **alleles** > from both of the chromosome pairs in diploid organisms are observed. Second, when used for individual identification—genotyping in genetic terms—each pair of bars on the barcode is a separate microsatellite locus. Thus, a **heterozygous** individual (an individual with two different alleles) would have two bars (called bands or fragments) from a single microsatellite (see samples 1, 2, 5, 7 and 8 in figure 9.1). Alternatively, a **homozygous** individual has only one fragment at the microsatellite locus (see samples 3 and 4 in figure 9.1). The ability to distinguish loci from one another enables traditional population genetic models to estimate phenomena such as gene flow or relatedness (Wright 1969). Third, microsatellites are believed to be selectively **neutral**, conforming to many population genetic models. These properties, plus the ability to either inexpensively develop microsatellites for a particular species or use those already developed for related taxa, have made microsatellites a popular tool for molecular ecologists studying wildlife.

In summary, the coupling of PCR and microsatellite or mtDNA primers allows small amounts of DNA (e.g., from cells attached to the follicle of a single hair) to be transformed into a diagnostic identifier of individuals, species, and populations and makes noninvasive genetic sampling feasible.

Wildlife Genetics in Practice: A Hypothetical Example

Imagine a genetic sampling survey with the goal of estimating carnivore species diversity in a western forest. Samples in this hypothetical survey consist of feces (also called scat, pellets, dung, or turds, depending on the publication) located by scat detection dogs (chapter 7) and hair snared at bait stations (chapter 6). In this section, we walk through the different analyses that are commonly conducted on such samples. It is important to note, however, that the particular molecular genetic techniques applied will depend on the objectives and species under study, as well as the expertise of the laboratory. One size doesn't fit all in molecular ecology.

Species Identification

The first question of interest to wildlife researchers is often, what species were detected by my survey? There are several ways in which a laboratory can ascertain species identification, but one of the most common is to **sequence** a region of the mitochondrial genome. For identifying carnivore species in particular, a standard approach is to use the PCR reaction with primers for the 16S rRNA region of the mitochondrial genome (following the protocols in Hoezel and Green 1992; Mills et al. 2000). Most North American carnivores have a distinct sequence at the 16S rRNA region, and the majority of these

species' sequences are entered into a national database (GenBank; National Center for Biotechnology Information 2007), which facilitates identification by matching sequences. For carnivores outside of North America, however, and for other taxa, reference sequences may not be available—although this is changing rapidly.

DNA barcoding is a new trend in molecular biology for species identification. With this approach, short, standardized DNA sequences—typically from a mitochondrial gene—are used to identify known species and to discover new species quickly and easily (Herbert et al. 2004; Savolainen et al. 2005). The initial goal of barcoding was to use a standardized region of the mitochondrial genome to uniquely identify all species, although this is proving difficult. Regardless, the barcoding databases established for many taxa will aid in developing noninvasive surveys designed for carnivore species' detection worldwide.

DNA sequencing can be expensive. In some cases, however, we can reduce expenses approximately 35% by using a restriction enzyme test to ascertain species identification. Here, as in sequencing, we amplify the 16S rRNA region, but we then immerse the DNA in particular enzymes which cut the DNA at diagnostic "restriction sites." We can identify species by examining the patterns of restriction enzyme-digested, PCR-amplified mtDNA (figure 9.2). This was the approach taken with the thousands of samples collected in the USDA Forest Service's National Lynx Survey (McKelvey et al. 1999; see chapter 6). The downside is that research is required to develop such assays. Further, when nontarget species are encountered, they can often not be identified to the species level and may even confound the identification of the target species. Given our hypothetical survey, with the goal of identifying every species that deposited a sample, we would likely sequence either 16S rRNA or another region of the mitochondrial genome and compare these sequences to known species sequences in a genetic database (e.g., GenBank). But if we only wanted to know whether or not the sample was deposited by a given target species, we might choose a restriction enzyme test (e.g., Paxinos et al. 1997; Mills et al. 2000; Dalen et al. 2004).

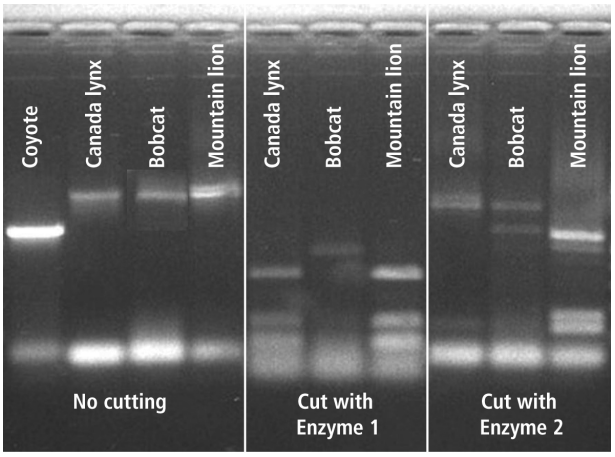

Figure 9.2. An electrophoresis gel showing the results of a restriction digest test to determine species identification. Without deploying any restriction enzymes, separation of felids and canids is possible. Using two different enzymes, Canada lynx, bobcats, and mountain lions can be discerned.

Gender Determination

Let's suppose that laboratory results show our hypothetical survey to have detected ten gray wolves (*Canis lupus*), eight Canada lynx (*Lynx canadensis*), four fishers (*Martes pennanti*), one elk (*Cervus elaphus*), and one bushy-tailed woodrat (*Neotoma cinerea*). The forest manager may want to know if there are any female lynx or gray wolves present in the forest (i.e., to assess whether these might be breeding populations). One of three genes is typically used to identify gender in carnivores. The first is the SRY gene (the *testis determining factor*), present only on the male Y chromosome. When a sample from a male is analyzed with SRY-specific primers, one band appears on an electrophoresis gel. If the sample is from a female, no bands appear. Unfortunately, a negative result (i.e., no band) can mean either that the sample originated from a female, or that it was of low quality and did not contain adequate amounts of DNA. Therefore, it is common for researchers to

coamplify a microsatellite locus with the *SRY* gene, which amplifies regardless of gender. Failure of the microsatellite to amplify signals that the DNA was of poor quality and the results should be discarded. Multiple repeats of this process are recommended for accuracy.

A second method for identifying sex is to sequence a gene in the *zinc-finger region* (*ZF*) of the X and Y chromosomes. In felids, the *ZFY* (male) band has a three-base pair deletion compared to the *ZFX*. Thus, a male lynx—and males of most other mammal species—will show two bands on an electrophoresis gel (i.e., a band for the X chromosome and a band for the Y chromosome, which vary in length because of the deletion on Y), whereas a female will only show one band (i.e., females have two X chromosomes, with no length variants; Pilgrim et al. 2005). Similar tests have been published for canids, cetaceans, and bovids. Last, a similar gene, called the amelogenin gene (which codes for proteins found in tooth enamel) has a twenty-base pair deletion on the Y chromosome of felids (and some other species), providing another gender determination test that works for felids (Pilgrim et al. 2005) as well as ursids (Poole et al. 2001).

Individual Identification

Our hypothetical research reveals that, of the eight lynx samples, five were produced by males and three by females. The next common question might be, how many individuals are represented by these samples? While several tools exist to provide individual identification, the most common are microsatellites (see figure 9.1). For lynx, a panel of six microsatellites is frequently used to determine individual identification (Schwartz et al. 2004). In a lynx study in Minnesota, six microsatellites provided a **probability of identity** of 1.55×10^{-06} (M. Schwartz, unpubl. data), which translates to the probability of two randomly chosen lynx in the Minnesota population having identical **genotypes** as being 1 in 645,161. Given that surveys to date have detected fewer than two hundred lynx in Minnesota, the survey was deemed to have had sufficient power to distinguish individuals with six microsatellites. The number of microsatellites necessary for individual identification depends on the amount and distribution of genetic variation in the species (characterized by the probability of identity; Waits et al. 2001). In other work, as few as four microsatellites, or as many as ten, have been required to achieve a reasonable probability of identity, depending on the population and its history (e.g., small and inbred populations tend to have little variability).

In cases where existing microsatellites have low variability, one solution can be to develop microsatellites specifically for the population of interest. Given that microsatellites show an ascertainment bias (i.e., they are more variable in the species and, in some cases, the population for which they are developed), this approach can result in variable microsatellites for the target population, thus requiring fewer microsatellites for individual identification. Today, a number of commercial companies can quickly develop variable microsatellites for a target population at a reasonable cost (e.g., $10,000–$15,000 USD).

The decision to develop microsatellites for a particular species, versus assessing whether suitable primers have already been developed from a closely related species, lies in the initial costs of developing markers, the availability of markers for a related species, and the purpose of the project. For instance, a recent study seeking a panel of microsatellites for sampling mountain beavers (*Aplodotia rufa*) found that the lack of congenerics and the requirement for short microsatellites to be used with individual hairs dictated the development of microsatellites (Pilgrim et al. 2006).

Once sufficient power to discriminate between individuals is achieved, the resulting microsatellite genotypes can be compared to determine the number of unique individuals (figure 9.1). When employing microsatellites to identify individuals with noninvasively collected genetic samples, it is important that some method be used to ensure that the resultant data are error free (see box 9.1). It should be noted that other genetic tools can be used to

Box 9.1

A cautionary note on field and laboratory errors

Errors occur in all scientific disciplines; the critical issue is whether and how they are detected and reported. Researchers conducting noninvasive genetic sampling have openly acknowledged, and attempted to address, errors that occur in the laboratory. Such errors are of two general types: those associated with labeling samples, misreading labels, and misscoring electrophoresis gels—deemed *human errors*—and those that are inherent when using low-quality or low-quantity DNA samples, called *genotyping errors*. The two most common types of genotyping errors are **allelic dropout**—the preferential amplification of one of two alleles from a codominant marker—and **false alleles**, amplification products that mimic true alleles.

Genotyping errors can dramatically affect survey results, especially when estimating abundance via genetic sampling (Waits and Leberg 2000; McKelvey and Schwartz 2004a, b). For example, Creel et al. (2003) demonstrated that, without addressing genetic errors, the population size of gray wolves in Yellowstone National Park would be overestimated by 550%. Similarly, before error-checking, Schwartz et al. (2006) found a 28.1% overestimate in the number of unique genotypes of black bears in Northern Idaho. This bias is in contrast to the underestimation that can occur when researchers use too few microsatellites to determine individuals and thus mistakenly infer a single individual from samples obtained from two unique individuals (deemed the "shadow effect"; Mills et al. 2000).

There are four primary methods used to identify and remove genetic errors. The first is called the multitube approach (Taberlet et al. 1996). In this approach, each sample at each locus is run up to seven times to ensure genotype consistency. While multitubing will detect genotyping errors, it has the disadvantage of being expensive in terms of cost and sample use. That is, if a ten-locus genotype needs to be rerun seven times at each locus, seventy runs of the sample are required. Sometimes there is not enough DNA for this approach (depending on the initial sample), or no DNA remains for future analyses. Thus, a small cottage industry has developed with the aim of finding less time-consuming, costly, and DNA-intensive methods to detect and remove errors.

The second approach is to quantify the amount of target DNA in the sample. Morin et al. (2001) developed an assay designed to measure the amount of amplifiable nuclear DNA in low DNA-concentration extracts. This method provides an indication of the concentration of DNA in the sample prior to any analyses and allows researchers to discard the sample or estimate the optimal number of times each sample should be analyzed to eliminate errors (see Morin et al. 2001 for details). While this approach undoubtedly reduces errors, it has two shortcomings. First, even if a relatively large amount of DNA is present, not all genotyping errors are caught. Second, the equipment required to measure target DNA remains uncommon in many laboratories, although this is changing rapidly.

A third method for addressing genotyping errors uses computer algorithms to detect them. Depending on the data and objective, various algorithms have been suggested. For capture-recapture data, McKelvey and Schwartz (2004a, b) developed two tests that were incorporated into program DROPOUT (www.fs.fed.us/rm/wildlife/genetics/software.php; McKelvey and Schwartz 2005) to detect both human and genotyping errors. The first test utilizes a longer genetic tag (e.g., a genotype that produces individual identification) than is typically used in capture-recapture studies and examines the distribution of differences in loci between all samples. The second test proposed by McKelvey and Schwartz (2004a, 2005) assesses the number of loci required to provide enough power to distinguish individuals (e.g., five loci), employs a greater number than this number (e.g., nine loci), and then runs through different combinations of five-locus genotypes to determine the number of unique individuals. If a locus has errors, its use in creating a genotype will result in an inflation of the number of unique individuals.

Another commonly used algorithm for detecting genotyping errors is the maximum likelihood-based method contained in program RELIOTYPE (Miller et al. 2002). This approach minimizes errors by estimating genotype reliability and directs which samples should be multitubed to remove errors. In other words, RELIOTYPE uses the allelic frequencies from a population and creates maximum likelihood estimates of the allelic dropout rate per locus (Miller et al. 2002). Those loci estimated to have the most errors can then be reanalyzed. Additional programs include GIMLET (Valiere 2002),

Box 9.1 (Continued)

which is useful for evaluating errors when samples have been multitubed (Taberlet et al. 1996); PEDMANAGER (Ewen et al. 2000), applicable when pedigree information is available; MICRO-CHECKER (Van Oosterhout et al. 2004), which compares randomly constructed genotypes to observed genotypes in order to determine scoring errors due to stutter and short allele dominance, and HW-QUICKCHECK (Kalinowski 2006), which uses exact tests to detect departures from Hardy-Weinberg proportions—a sign of genotyping error. Relatively little research has been conducted to evaluate the effectiveness of one approach versus another (but see Smith et al. 2006).

The final approach to handling genotyping errors is used solely in capture-recapture studies (Lukacs and Burnham 2005a). This method incorporates the probability of genotyping error into the closed-population models of Otis et al. (1978), Huggins (1989), and Pledger (2000) by analyzing the disproportionate number of genotypes collected once relative to genotypes collected more frequently. While approaches that incorporate error into estimates have potential value, they have only recently been developed and have not been widely used or thoroughly evaluated with actual datasets (for further discussion of genotyping error, see chapter 11).

distinguish individuals (see Avise 2004 for a description of these techniques)—with each having its own benefits and limitations.

Inference to Populations

Our hypothetical forest manager might need information about the population composition (e.g., origin of a reintroduced population, population membership) of fishers found in the forest. For example, fishers in the Rocky Mountains are either descended from animals reintroduced between the 1950s and the 1990s or from a remnant population that escaped extinction (Vinkey et al. 2006). Reintroduced fishers have a unique mitochondrial DNA signature at both the **cytochrome-b** region (mtDNA) and the **control region** (mtDNA), compared to native individuals. We could thus analyze the four fisher samples from our hypothetical survey to determine the historical maternal origins of the animals from which the samples were taken (remember that mitochondrial DNA is transmitted only through mothers, thus nothing can be inferred about the paternal lineage with this approach). Alternatively, we might be able to use molecular markers, such as microsatellites, to assign individuals to a population (if reference databases are available), or even to classify and subsequently assign subspecies or other taxonomic designations (for a review of genetic tools and additional types of analyses for determining subspecies, see Baker 2000; Avise 2004; Allendorf and Luikart 2007; Palsbøll et al. 2007).

Types of Genetic Samples

DNA analysis has been attempted on biological samples ranging from historical pelts to regurgitates found on the side of a trail (table 9.2), but hair (Morin and Woodruff 1992; Taylor et al. 1997) and feces (Taberlet et al. 1997; Prugh et al. 2005) are the most common sources of noninvasive genetic material collected for wildlife research and monitoring (table 9.1). In one of the first applications of noninvasive genetic sampling, Taberlet et al. (1997) used a combination of hair and feces collected in the field to track a small population of Pyrenean brown bears. After extracting DNA from the samples, the authors were able to amplify six variable microsatellite loci and obtain individual and gender identification information that showed the population consisted of at least five bears: four males and one female.

The target for sampling hair is the follicle located at the end of the hair shaft; follicles are larger on guard hairs than on underfur. Occasionally, hair

Table 9.2. Source material typically used for genetic sampling

Source Material	*Species*	*Purpose*	*Reference*
Hair	Chimpanzee (*Pan troglodytes*)	Paternity and gene flow estimates	Morin et al. 1994
	Brown bear	Abundance	Mowat and Strobeck 2000
Feces	Coyote	Population size estimation	Kohn et al. 1999 ; Prugh et al. 2005
	Eurasian badger (*Meles meles*)		Frantz et al. 2003
Regurgitates	Gray wolf	Documentation of dispersal	Valiere 2003
Saliva	Coyote	Predator identification	Williams et al. 2003; Blejwas et al. 2006
	Wolf		Sundqvist et al. 2008
Urine	Wolverine	Methodological study	Hedmark et al. 2004
Menstrual bleeding	Taiwan macaque (*Macaca cyclopis*)	Microsatellite development	Chu et al. 1999
Sloughed skin	Humpback whale (*Megaptera novaeangliae*)	Abundance estimation	Palsboll et al. 1997
	Ringed seal (*Phoca hispida*)		Swanson et al. 2006
Blood in snow	Wolf	Species identification	Scandura 2005
	Multiple felids	Species identification	M. Schwartz, unpubl. data
Museum specimens	Wolverine	Evolutionary significant units	Schwartz et al. 2007
	Brown bear		Leonard et al. 2000
Prey amplification	Prey from carnivores	Diet	Farrell et al. 2000

samples without follicles provide positive DNA—but not reliably. As a source of DNA, one advantage of hair over scat is that hair contains fewer chemical inhibitors. Furthermore, contamination from other DNA sources (e.g., prey DNA found in scat) are minimized with hair (although allogrooming and other social behaviors may cause cross-contamination). There is often a high rate of success in determining individual identification from hair (e.g., Frantz et al. [2004] had a 93% success rate with European badgers [*Meles meles*])—although this varies by study. Published success rates vary from 15% to greater than 90%.

Alternatively, fewer cells—and less total DNA—are generally available in a hair sample than a scat sample. Thus, unless a clump of hair is obtained, hair samples are often expended after a single DNA extraction; failure to obtain DNA leaves no opportunity for a second extraction attempt. Prior to launching a survey, it is highly advisable to conduct a pilot study to determine the rate of success of obtaining DNA from the hair of the target species under normal survey conditions (Goossens et al. 1998). The large variation in success is due to such factors as the morphological characteristics of the species' hair, the social characteristics of the species, the environmental conditions under which the sample is collected, storage and laboratory methods, the goals of the study, and the quality of results accepted by the researcher and the laboratory.

One practical consideration is whether to consider a clump of hair as having originated from a single or multiple individuals. The answer will likely depend on many variables, including the life history characteristics of the species and the goal of the study. If the goal is species detection, the laboratory can often identify which species are represented in a mixed sample. Laboratories can often detect if multiple individuals were sampled, but they can do little to recover information regarding *which* individuals were present in a mixed sample (Alpers et al. 2003; Roon et al. 2005). Alpers et al. (2003), however, note that if there are few alleles in a population, there will be times when mixed samples will not be accurately identified as having come from multiple individuals. Instead, "new" individuals will be erroneously

"created" by the combined genotype profile of multiple individuals. Thus, if individual identification is indeed a goal, researchers should attempt to minimize collecting samples from multiple individuals. This might be accomplished by frequently revisiting hair collection devices, or by using single-catch hair collection methods (e.g., Belant 2003a; Bremner-Harrison et al. 2006; also see chapter 6).

Advantageously, feces contain many sloughed epithelial cells. In addition, most carnivore fecal samples are large enough to allow multiple attempts at DNA recovery. Last, there is usually relatively little ambiguity as to the number of individuals that deposited fecal samples, although overmarking by conspecifics or sampling from latrines can potentially produce cross-individual contamination.

The greatest constraints to fecal analysis are the chemical inhibitors present in feces that restrict the amplification of DNA. Also, amounts and quality of fecal DNA are known to vary by species, temperature at time of collection, age, season, preservation method, diet, storage time, and extraction protocol (Murphy et al. 2002; Piggott and Taylor 2003; Maudet et al. 2004; Nsubuga et al. 2004; also see table 9.3). As a result, rates of species and individual identification from feces are extremely variable. McKelvey et al. (2006), for example, were 100% successful in identifying species from lynx feces collected in Washington, although individual identification rates were significantly lower (K. Pilgrim, USDA Forest Service, pers. comm.). Bellemain et al. (2005) report a 70% individual identification success rate with brown bear fecal samples at six to seven microsatellite loci, including a locus diagnostic for gender.

There has been relatively little study of the success of obtaining DNA from urine, regurgitates, saliva, or menstrual blood. In our experience, these materials have proven suboptimal sources of DNA compared to hair and feces, are difficult to systematically sample, and are better left to opportunistic collecting (but see Hedmark et al. 2004, who reported 40% success in determining individual identification with wolverine [*Gulo gulo*] urine as compared to a 65% success rate with feces). Regardless of the sample type, the way in which it is treated in the field will drastically affect the effectiveness of the survey.

Sample Treatment and Preservation

Almost all wildlife genetic studies require complex and expensive field operations to obtain samples. In fact, once field personnel, transportation, equipment, housing, communications, and other field costs are accounted for, laboratory costs usually pale in comparison. In many instances, field data are meticulously collected, yet samples are treated improperly or are inadvertently contaminated. Below we discuss the handling and treatment of field samples.

Contamination

Contamination is a major concern for many noninvasive studies (depending on the objective), and can occur in the field or laboratory. In the field, for example, contamination can be caused by baits, lures, previously handled animals, accompanying pets, or field personnel. Considering that the target sample may comprise only a few cells at the end of a hair, it is important to limit contact with material that can mask the target sample. We recommend the use of new latex gloves and sterile mechanical devices (e.g., tweezers, wooden picks) for handling all samples in the field. Gloves should be changed between the handling of different samples, and mechanical devices can be sterilized with ethanol and a lighter, washed in a weak bleach solution, or replaced between samples. Some of these safeguards can be diminished depending on the research question at hand. For instance, if microsatellites are to be used to determine the individual identification of nonprimates, contamination from field personnel is less of a concern—although cross-sample contamination must still be guarded against. But contamination can be an issue for studies that use universal genetic tools (e.g., mitochondrial DNA to identify species for estimating occupancy, or gender-specific markers to identify sex). Given that the goals of many studies

Table 9.3. Summary of fecal preservation methods used in seven recent DNA-based studies

			Method												
Reference	*Species*	*DNA type*	*DET*[a] *solution/ buffers*	*70% EtOH*[b]	*90%–100% EtOH*[b]	*Frozen –20°C*	*Air dry*	*Freeze dry*	*Oven dry*	*Micro-wave*	*Silica desiccant*	*Oven/ silica*	*Oven/ stored –20°C*	*EtOH/ silica*	*Drierite*
Frantzen et al. 1998	Baboon[c] (w)	Mt	2	4		3	1								
Frantzen et al. 1998	Baboon (w)	N	1	3		3	2								
Murphy et al. 2000	Brown bear (c)	Mt						1	2	3	4				
Murphy et al. 2000	Brown bear (c)	N						1	2						
Murphy et al. 2002	Brown bear (c)	Mt	1		1						5	4	3		
Murphy et al. 2002	Brown bear (c)	N	2		1						5	4	3		
Wasser et al. 1997	Black bear (w)	Mt	4	6	5			2			1				3
Wasser et al. 1997	Black bear (w)	N	4	5	5			2			1				3
Nsubuga et al. 2004	Mountain gorilla[d] (w)	D[e]	3								2			1	
Roeder et al. 2004	Mountain gorilla (c)	D[c]			2						3	1			
Roeder et al. 2004	Mountain gorilla (c)	Mt			2						2	1			
Piggot and Taylor 2003b	Tasmanian pademelon[f] *Thylogale billardierii* (c)	N	4	2		2	1								

Note: Numbers in cells are rank orders (with 1 being highest) of the effectiveness of each method within each study based on our interpretation of tables and discussions in the original work. Studies that examined the influence of preservation methods on both mitochondrial DNA (Mt) and single-copy nuclear DNA (N) are featured in two respective rows. Each paper included caveats for field conditions, duration of storage, and discussions as to why the particular method was likely superior—see original publications for specific details. In the species column, (w) and (c) denote studies of samples collected from wild and captive animals, respectively.

[a]DET is a DMSO salt solution comprised of 20% DMSO, 0.25 M sodium-EDTA, 100mM Tris, pH 7.5, and NaCl to saturation (Seutin et al. 1991).

[b]Ethanol

[c]*Papio cynocephalus ursinus*

[d]*Gorilla gorilla*

[e]Quantity of target DNA

[f]*Thylogale billardierii*

change over time, we recommend implementing protocols that minimize all types of contamination.

Every laboratory has protocols in place for detecting contamination (see box 9.2 for what to consider when choosing a laboratory), and laboratories that routinely process noninvasive genetic samples have separate facilities for receiving and extracting DNA. In addition, the bleaching or UV irradiation of laboratory surfaces is routine. Furthermore, laboratories specializing in noninvasive genetic sampling may limit access to areas where noninvasive samples are analyzed and discourage technicians from entering a main laboratory before processing samples in the satellite facility. Finally, all laboratories will routinely run both positive and negative controls (e.g., samples comprising simply distilled water) to detect laboratory contamination. While such measures minimize contamination from other samples or PCR products found within the laboratory, even the most stringent lab will unlikely be able to discern field contamination (e.g., the cross-contamination of samples between hair snares as a result of improper handling).

Preventing Sample Deterioration

Given proper storage conditions, DNA is a robust and stable molecule that can persist for thousands of years (e.g., Hofreiter et al. 2001; Leonard et al. 2005). The main adversaries of DNA are **hydrolysis**, oxidation, physical cleavage through freeze-thaw cycles, alkylation, and UV radiation. Most storage techniques are designed to halt the principal enemy of DNA—hydrolysis—by eliminating water from the sample either through chemical or physical drying. Placing a sample in a silica desiccant or in an oven mechanically dries the sample, thus minimizing degradation. Alternatively, depositing a sample in ethanol or a buffer solution chemically dries the sample.

There have been a multitude of studies to examine the best way to minimize deterioration of fecal samples (table 9.3). These studies have compared the integrity of samples preserved by the following methods:

1. Drying at room temperature or in a warm room, oven, or microwave, and storing dry.
2. Drying and storing in 70%–100% ethyl alcohol.
3. Freezing at –20°C.
4. Saturating and storing in a buffer solution.
5. Drying and storing in a silica- or Drierite-based desiccant.
6. Drying in a lyophilizer (i.e., a freeze dryer).
7. Drying with an oven or ethanol, then storing with silica desiccant.

Most of these studies have been limited to only a few methods applied to samples from one species. The most striking finding from our comparison of results (table 9.3) is the lack of consistency between studies. For example, silica desiccant proved to be the best storage mechanism for black bears (Wasser et al. 1997), and the worst for brown bears (Murphy et al. 2002). Similarly, storage in ethanol performed poorly for Frantzen et al. (1998) and Wasser et al. (1997) but was the second best storage system for Piggot and Taylor (2003). These discrepancies are likely due to factors relating to the species (e.g., omnivores versus carnivores, species with high-lipid versus low-lipid diets), environmental conditions (e.g., mesic versus xeric, many freeze-thaw cycles versus constant cold), field and laboratory protocols (e.g., duration of storage, speed of sample drying, laboratory extraction technique, dessication protocols), and study objectives (e.g., individual versus species identification). Piggott and Taylor (2003) noted an interaction between storage method and extraction technique in the laboratory (i.e., certain extraction techniques performed better with certain storage methods, and vice versa). Again, these results strongly support conducting a pilot study to explore the performance of various storage and extraction techniques.

Given such varied study results, it is difficult to make sweeping recommendations as to the best way to store fecal samples. Here are some general rules of thumb:

Box 9.2

Choosing a DNA laboratory

We recommend selecting a laboratory at the beginning of a survey, and working closely with this lab for the duration. While much of the equipment used in various labs is the same, the interests and expertise of each lab is slightly different. Some labs are well equipped to conduct noninvasive genetic sampling projects, while others are not. And while some labs have collaborated with dozens of noninvasive projects and employ experienced technicians to handle the anomalies that arise during such efforts, others are less experienced. Finally, certain labs will conduct or assist with post-genotyping statistical analyses (e.g., assignment tests, tests for genetic structuring).

Although there are many factors to consider when choosing a lab as a partner, most project managers overemphasize cost at the expense of other considerations. We propose ten questions that a researcher should address prior to choosing a lab:

1. Has the lab worked with the type of samples (e.g. hair, scat) that will be used in this particular survey? Many laboratories have little or no experience with noninvasive genetic samples, nor do they have a separate lab to conduct DNA extractions that will reduce the risk of contamination.
2. Has the lab conducted analyses for surveys of similar size and scale?
3. Has the lab worked with your target species and employed the particular genetic tools you prefer?
4. How experienced are the technicians? Commercial labs employ technicians with many years of experience, whereas universities sometimes rely on relatively untrained students. While there are benefits to training students, there also may be costs in terms of quality. Further, because of academic calendars and the demands of a student's own work, timelines for the delivery of genetic results may be difficult to predict. Agency labs are another option, but they often focus solely on projects central to the agency's mission.
5. Are you looking only for lab results, or are analysis and interpretation also important?
6. Over what time frame is the project scheduled? As genetic monitoring approaches become more common, long-term studies will also increase in number. Using one lab consistently prevents errors that may result from changing labs (and therefore protocols and technicians). Furthermore, many data types (e.g., those produced by microsatellites) are relative—versus absolute—measures. Changing labs will require both the former lab and the new lab to calibrate initial results if data are to be analyzed over time.
7. Can the lab store your samples over time? This may be important if you need to run additional analyses in the future.
8. How does the lab check for errors? Is the lab willing to re-run samples that contain potential errors? Error-checking has become an important aspect of genetic analyses, and different labs are likely to approach this topic in different ways (see box 9.1).
9. Are lab costs competitive given the services and quality offered?
10. If the samples or results are contentious and could end up presented in court, the following questions may also apply:
    - Does the lab have forensic certification or follow forensic protocols?
    - Has anyone from the lab served as an expert witness in a trial?
    - How many people have access to the lab?
    - Are samples secured? Is the lab secured?

Finally, we recommend confirming who will own the resulting data, and to whom and by whom they can be disseminated? Posing these questions early on can eliminate contention later in the research process.

1. *Extract early*. Almost all studies that have examined sample quality in relation to time have demonstrated a deterioration of DNA (Roon et al. 2003). It may be useful to send samples to the laboratory (see box 9.3 for instructions on labeling, tracking, and shipping samples) and to have DNA extracted throughout the duration of the survey—even if the survey's exact objectives are still undetermined. DNA should persist longer in a laboratory buffer than in feces.
2. *Pilot studies*. Whenever possible, a pilot study should be conducted to test storage methods and extraction procedures. For example, feces collected in a captive setting can be subjected to various conditions for varying lengths of time and stored using several different methods to establish species-specific protocols.
3. *Imitate success*. If a pilot study is not possible, consider the species, its diet, the size of the sample, the environment, the laboratory, and laboratory extraction methods. Choose a storage technique that has been successful in other studies with similar conditions.
4. *The devil is in the details*. When investigating a storage protocol with a proven track record, research the specific products used (e.g., Fisher brand silica desiccant, mesh size 10–18, part number S161212) and the precise details of the protocol. Ethanol varies in concentration and contains contaminants added to prevent human consumption. Similarly, silica desiccant varies by mesh size, and the results of air drying differ with field technician accommodations. It is also important to understand protocol details in terms of absolute amounts of sample collected, ratios of sample to ethanol or silica desiccant (e.g., 5 ml ethanol /1 g feces), container sizes (e.g., surface area exposed), and an approximate rate at which the sample will dry.
5. *Field conditions matter*. Even the *best* storage system is useful only if it can be effectively implemented in the field. Asking a technician who lives in a tent to air dry a sample may not be realistic, even if air drying is deemed the most effective protocol. Silica desiccant is often used in these situations, as it doesn't leak, it is easily portable, and it often contains an indicator chemical that changes color when it is saturated with moisture.

Less is known about hair preservation than fecal preservation. Roon et al. (2003) compared storing hair samples in silica desiccant with freezing them at –20°C. Although these researchers found no significant difference between methods used to preserve hair for mitochondrial DNA work, freezing was slightly—but consistently—better for microsatellite (nuclear) DNA tests. It should be noted, however, that this was a study of captive animals, thus enabling the freezing of samples upon collection. In some field studies, freezing samples at –20°C may not be an option until well after they are collected. If freezing at the field site is possible, it is important to avoid subsequent thawing in transit to the laboratory, as this process can mechanically cleave DNA—thus diminishing its quality. In field situations, we have found that storing hair either directly in silica desiccant or in paper envelopes in a silica desiccant dryer produces adequate results. To our knowledge, there is no information available to permit the comparison of storage methods for other types of noninvasively collected samples.

Pitfalls, Cautions, and Future Perspectives

No technology is a panacea; this certainly holds true for molecular markers. Although molecular geneticists can currently identify individuals, sex, and sometimes population membership, little information is available on an individual's age or life-stage (i.e., young-of-the year, juvenile, adult) from a noninvasive sample (although see Nakagawa et al. 2004 for future possibilities). To obtain some data for population demographic analyses, tags may need to be placed on animals that don't possess naturally unique identifying markings—often requiring physical capture of the animal.

Box 9.3

Labeling, tracking, and shipping genetic samples

It is critical to work with the genetics laboratory to accurately label and track samples, especially for larger studies. Each sample requires a unique and obvious identifier. While this may seem trivial, it is not uncommon to end up with vials having similar labels if multiple field crews are working concurrently (e.g., "sample collected 9/27/04, hair #1"). If samples arrive at the lab without clear and accurate documentation, confusion can ensue. Thus, we recommend that a designated field coordinator be assigned to organize all samples, ensure that each sample is assigned a unique identifier, compile a master list, and send all this information to the lab. Our second recommendation is to use a barcode system; a number of labs that process many samples have purchased bar code readers in recent years (see figure 6.16 for a description of barcode labeling).

From the perspective of the lab, the following information is helpful to include in a shipment of samples:

1 .*Sample list*. Many researchers include an electronic sample list and a print-out with their samples. Often, this list includes the field data associated with a sample (e.g., location, collector, comments). After genotyping a sample, genetic data is entered into the initial electronic sample list. This reduces transcription errors and errors associated with manipulating spreadsheet files at the lab.
2. *Copies of the necessary permits*. Many samples are collected under state, national, and international permits. Most labs maintain a file of these permits. Additional permits (e.g., CITES permits) may be necessary if samples are sent to a lab outside the country of collection.
3. *Chain of custody form*. If samples are potentially contentious, a chain of custody form should be completed to track access to each sample.

There are many other cautions that need to be heeded before conducting a molecular genetic study. A frequent mistake made by many researchers is to assume that simply sending noninvasive samples to a laboratory will yield answers to all questions of interest. It is not unusual for someone to send samples to a laboratory and to expect a report without ever having posed a question or explicitly described the desired data. It is even more common for wildlife researchers to underestimate the effort required to conduct an analysis for a given project; after all, on television, human forensic samples are analyzed between commercials. For instance, most biologists are aware that molecular markers can determine parentage (to estimate the relative abundance of offspring in a sample), yet it is frequently assumed that this is a trivial exercise. Often, numerous molecular markers are required to provide adequate power for assigning paternity and maternity simultaneously—sometimes more than are readily available or affordable. By comparison, determining paternity given known maternity (or other information acquired in the field) is far less intensive. Thus, combining field data with genetic data can save analysis time and money. These same caveats hold true for a suite of other questions, including those related to estimating absolute abundance and distribution.

On the positive side, molecular genetic methods are advancing quickly. In the foreseeable future, additional molecular tools such as microarrays—which allow the examination of hundreds of loci at one time—and **single nucleotide polymorphisms** (SNPs; see Luikart et al. 2003), another type of molecular marker, may enable more information to be obtained from genetic samples. In fact, SNPs may ultimately replace microsatellites as they have the advantage of better conforming to well-characterized models of evolution and are more common throughout the genome (Aitken et al. 2004; Seddon et al. 2005). Furthermore, unlike microsatellites, which yield relative scores that require standards to be used for comparing results between laboratories,

SNPs are believed to provide data with absolute scores—thus facilitating collaboration between researchers studying the same species. To date, the expense of developing SNPs, and questions regarding error rates, ascertainment biases, their effectiveness with noninvasive samples, and within-population variability, have limited their use in conservation genetics (Morin et al. 2004). But these issues will likely soon fade (Kohn et al. 2006; Morin and McCarthy 2007).

Rapid developments in the field of molecular ecology will continue to advance how noninvasive genetic sampling can be used to estimate abundance and occurrence. To maximize the utility of the approaches used, close collaboration between laboratory and field biologists must continue and improve. Field biologists should understand the limits of their data, while laboratory biologists must develop new tools with field applications in mind. Genetic sampling—although simple in principle—is actually complex in its execution, with attention to detail required from survey design through data analysis. Fortunately, there has been significant interest in this area, and the resulting research has demonstrated our ability to use noninvasive genetic sampling to monitor and study wild carnivore populations.

Endocrine Approaches for Studying and Monitoring Carnivores

Yallow and Berson (1959) were awarded the Nobel Prize for developing the first **immunoassay** for assessing minute concentrations of hormones (i.e., 10^{-12} gm/ml) in blood circulation. These methods were initially adapted for wildlife to measure *steroid hormones* in urine from diverse primate species (Hodges et al. 1979). **Steroid hormone metabolites**, quantified "noninvasively" in excreta (urine or feces), permit wildlife biologists to study reproduction and stress physiology in individuals, populations, or species, without disturbing animals (Lasley and Kirkpatrick 1991; Monfort 2003).

Urinary (in nonhuman primates; Hodges et al. 1979; Andelman et al. 1985) and fecal (in domestic livestock; Bamberg et al. 1984; Möstl et al. 1984) steroid monitoring techniques, pioneered with captive animals, have been adapted to study diverse biological phenomena in free-ranging animals, including reproductive seasonality, gonadal status, pregnancy rates and age-specific fecundity, and the endocrine mechanisms controlling reproductive fitness in social mammals. Adrenal glucocorticoid metabolites (GCs) were first used as a proxy for "stress" in wildlife studies of bighorn sheep (*Ovis canadensis*; Miller et al. 1991). Urinary and fecal GC metabolites have since been used to evaluate physiological stress associated with social status, and the effects of environmental disturbance on animal well-being and fitness (Goymann et al. 1999; Creel et al. 2002; Sands and Creel 2004; Young et al. 2006).

A Primer on Endocrine Tools: Measures for Assessing Reproduction and Stress

Noninvasive endocrine measures avert the physiological stress resulting from animal capture, restraint, and/or anesthesia, allowing normal underlying hormonal patterns to remain undisturbed. Additionally, unlike blood samples, which yield a single "point in time" measure of endocrine status, urine and feces provide an integrated measure of hormone production (i.e., from hours to days) due to the preexcretion 'pooling' that occurs in the urinary bladder—or, in the case of feces, in the intestinal tract (Millspaugh and Washburn 2004). Pooled hormone measures dampen episodic secretory patterns that normally occur in blood circulation—a potential benefit when seeking to evaluate overall patterns of hormone production (Monfort 2003). Endocrine methods permit repeated longitudinal sampling in individuals, as well as sampling across populations to facilitate population-scale studies.

With the exception of estrogens (i.e., estradiol, estrone), little unmetabolized steroid (e.g., progesterone, testosterone, corticosterone) is excreted in urine and feces. This is because gonadal and adrenal

steroids circulating in the bloodstream are metabolized in the liver and/or kidney before excretion into urine or bile (which is delivered to the gastrointestinal tract and eliminated via defecation), and rendered biologically impotent during metabolism through subtle molecular changes and/or through the attachment (conjugation) of highly charged side chain molecules that increase water solubility to facilitate excretion (Taylor 1971).

The proportion of metabolites derived from any given class of steroids (i.e., estrogens, androgens, progestagens, or corticosteroids) excreted in urine or feces is species- or taxon-specific, ranging, for example, from > 90% fecal excretion in felid species (Brown et al. 1994) to > 80% steroid excretion in baboon (*Papio cynocephalus cynocephalus*) urine (Wasser et al. 1994). To complicate matters further, some species excrete one class of steroid molecule into feces (e.g., progesterone metabolites), whereas another class (e.g., estrogen metabolites) may be excreted predominantly in urine (e.g., Sumatran rhinoceros [*Dicerorhinus sumatrensis*], Heistermann et al. 1998; African elephant [*Loxodonta Africana*], Wasser et al. 1996). In all species, there is a variable **excretion lag-time** between when steroid production and secretion occur in the bloodstream and byproducts appear in excreta, ranging from < 12 hours (e.g., African wild dog [*Lycaon pictus*]; Monfort et al. 1997), 12–24 hours (e.g., scimitar-horned oryx [*Oryx dammah*]; Morrow and Monfort 1998), and 24–48 hours in primates (e.g., baboons; Wasser et al. 1994) and colon or hindgut fermenters (e.g., elephants; Wasser et al. 1996).

Steroid metabolites can be quantified using a variety of immunoassays (e.g., radioimmunoassay, enzyme immunoassay, fluorescent immunoassay) techniques. In general, immunoassays that employ a broad spectrum or "group specific" antibody that cross-reacts with a host of similarly structured steroid metabolites within a given class of steroid (e.g., estrogens, progestagens, androgens, corticosteroids) are preferred for assessing steroid metabolites in excreta, and literally dozens of different commercially available or custom-made immunoassays have provided suitable results (Lasley and Kirkpatrick 1991; Brown et al. 1994; Wasser et al. 1994, 2000; Schwarzenberger et al. 1996; Monfort 2003; Young et al. 2004; Heistermann et al. 2006).

Regardless of the immunoassay technique employed, each assay must be validated for each new species or biological fluid to demonstrate that it yields reliable and consistent estimates of hormone production (Niswender et al. 1975; Reimers et al. 1981). It is particularly important to demonstrate that measured hormone concentrations provide physiologically relevant information. For example, an ovarian cycle might be validated by (1) comparing two independent measures of the same hormone in matched samples (i.e., fecal versus urinary estrogen); (2) comparing temporal hormone excretion patterns with external signs of reproductive status (e.g., sex skin swelling, copulatory or rutting behavior); (3) demonstrating cause-and-effect patterns, such as a rise and fall in hormone concentrations coincident with pregnancy onset and parturition, respectively; or (4) analyzing gonadal or adrenal responsiveness to a challenge with *gonadotropin releasing hormone* [GnRH] or *adrenocorticotrophic hormone* [ACTH], respectively (Monfort 2003).

Endocrine Monitoring in Practice

Here we discuss two common applications for endocrine monitoring in the context of noninvasive wildlife research—those pertaining to reproduction and stress.

Reproductive Life History Strategies and Pregnancy Determination

In the DNA section above, we discussed a hypothetical survey that used molecular markers to individually identify eight lynx. One question posed was whether or not this was a breeding population. Through the use of molecular markers, we were able to determine that there were three females in this population, but very little could be ascertained about their reproductive status. Here we can now turn to endocrine biology, assuming a proper validation of

hormone levels has been conducted for the target species.

Endocrine monitoring has been extensively used to characterize reproductive status in free-ranging wildlife, including carnivores (Creel et al. 1992, 1995, 1997, 1998; Goymann et al. 1999, 2001; Clutton-Brock et al. 2001; Moss et al. 2001; Dloniak et al. 2004; von der Ohe et al. 2004; Wasser et al. 2004; Young et al. 2006), rodents (Billiti et al. 1998; Harper and Austad 2000; Touma et al. 2003), ungulates (Chaudhuri and Ginsberg 1990; Kirkpatrick et al. 1993; Foley et al. 2001; Pelletier et al. 2003), and especially primates (Wasser 1996; Hodges and Heistermann 2003; Ziegler and Wittwer 2005). In the case of primates, investigations have focused on establishing the basic interrelationships between hormones and behavior in both sexes (Brockman and Whitten 1996; Brockman et al. 1998; Strier and Ziegler 1997; Ziegler et al. 1997, 2000; Cavigelli 1999; Curtis et al. 2000; Herrick et al. 2000; Fujita et al. 2001; Lynch et al. 2002; French et al. 2003; Harris and Monfort 2003, 2005; Campbell 2004; Muller and Wrangham 2004a, b).

Furthermore, fecal progesterone metabolites have been used to assess pregnancy status in a variety of wildlife species (e.g., elk, Garrott et al. 1998; Stoops et al. 1999; moose [*Alces alces*], Monfort et al. 1993; Schwartz et al. 1995; Berger et al. 1999; horses [*Equus caballus*], Kirkpatrick et al. 1991b; bison [*Bison bison*], Kirkpatrick et al. 1993, 1996; bighorn sheep, Schoenecker et al. 2004; black rhinoceros [*Diceros bicornis*], Garnier et al. 1998); and meerkats [*Suricata suricatta*], Moss et al. 2001; Young et al. 2006). For example, fecal progesterone metabolites (Berger et al. 1999) were assessed to determine whether a reduction in moose numbers was the result of decreased fecundity associated with habitat degradation or of increased neonate predation by reintroduced wolves and grizzly bears in the Greater Yellowstone Ecosystem. Hormones confirmed that pregnancy rates were among the lowest of any moose population in North America, enabling biologists to conclude that neonate predation could not explain the observed decline in moose populations.

Stress Related to Social, Environmental, and Human Disturbance Factors

Urinary and fecal GC metabolites are being used to evaluate adrenal status in a growing number of wildlife species (Goymann et al. 1999; Wasser et al. 2000; Hunt and Wasser 2003; Millspaugh et al. 2003; Monfort et al. 2003; Harper and Austad 2004; Kuznetsov et al. 2004; Cavigelli et al. 2005; Gould et al. 2005; Mateo and Cavigelli 2005; Heistermann et al. 2006; Young et al. 2006). This application is based on the premise that stress hormone production—mediated by neural and psychosocial inputs from higher brain centers that stimulate the production and secretion of GCs from the adrenal cortex (Moberg 1985; McEwen 1998, 2000)—can be approximated by assessing GC metabolites in the excreta of free-ranging wildlife (Wasser et al. 2000). Glucocorticoid excretion patterns have been especially useful for evaluating the interrelationships between hormonal measures, dominance rank, age, genetic relatedness, reproductive status, and rates of aggression among members of each social group (Creel et al. 1992, 1996, 1997; Muller and Wrangham 2004a, b; Sands and Creel 2004; Creel 2005). In one study, it was shown that dominant African wild dogs (Creel et al. 1996, 1997) excreted elevated GC concentrations compared to subordinate pack members. Because dominant wild dogs were involved in more aggressive interactions—presumably to maintain dominance—it was hypothesized that stress may be a cost of social dominance (Creel et al. 1996).

Additionally, GC excretion has been used to investigate the impact of human disturbance on wildlife, including, for example, the influence of logging activities on Northern spotted owls (*Strix occidentalis caurina*; Wasser et al. 1997; Tempel and Gutierrez 2003, 2004); the effect of snowmobiling activity on wolves and elk (Creel et al. 2002); the stress of radio-collaring wild dogs (Creel et al. 1997); and the stress physiology of prerelease conditioning and reintroduction on whooping cranes (*Grus americana*; Hartup et al. 2005). These studies show the promise of GC assessments for studying

the relationships between environmental stressors and animal well-being. It is critical to emphasize, however, that there is a serious risk of oversimplifying and/or overinterpreting GC data by suggesting that elevations in fecal GCs, in and of themselves, signal physiological or psycho-social "stress." In some cases, elevated or reduced GCs may be completely normal, reflecting changes in metabolic or nutritional status, reproductive life history, pregnancy, seasonality, prehibernatory preparations, and myriad other adaptive physiological states (von der Ohe and Servheen 2002). Thus, elevated GCs alone are not necessarily an index of stress, and these measures should be assessed in concert with other prospective indicators—activity patterns, behaviors, body condition, disease status, immunocompetence—the sum total of which may signal that an animal is experiencing physiological or psycho-social stress.

Types of Samples for Endocrine Evaluations

Various types of samples can be used to conduct endocrine evaluations. Here we discuss urine and fecal samples.

Urine Samples for Endocrine Evaluations

Small amounts of urine can be rapidly absorbed into natural substrates, making this a challenging medium for the wildlife biologist to use in conducting endocrine evaluations. Nevertheless, urinary gonadal (indicators of reproduction) and adrenal steroid (putative indicators of stress) metabolites have been evaluated in a diversity of free-ranging primate, carnivore, and ungulate species (see review, Monfort 2003). For example, hormones have been assessed in urine collected directly off the ground (elephants, Poole et al. 1984; Przewalski's horses, Monfort et al. 1990; bison, Kirkpatrick et al. 1991a; gorillas [*Gorilla gorilla*], Robbins and Czekala 1997); from urine-soaked snow (feral horses, Kirkpatrick et al. 1991b) and Kalahari sand (meerkats, Clutton-Brock et al. 2001; Moss et al. 2001); by positioning an observer under arboreal primates until they urinated onto a piece of aluminum foil attached to the end or stick (Harris and Monfort 2003, 2005); and even from rubber 'flip-flop' sandals that were urinated on as part of gregarious scent-marking behavior (dwarf mongooses [*Helogale parvula*], Creel et al. 1992, 1995).

A major advantage of urinary hormone monitoring versus fecal steroid monitoring is that samples can be assayed without further processing, thus minimizing labor costs. Another advantage is that excretion lag-times are generally short relative to hormone secretion in blood circulation, and day-to-day fluctuations in fluid balance can be easily calibrated to creatinine excretion (Taussky 1954). In summary, urine samples are generally difficult to collect under field conditions, but the tradeoff is that laboratory procedures are simplified and relatively inexpensive.

Fecal Samples for Endocrine Evaluations

Reproductive and adrenal steroids have been assessed in feces collected from a range of free-living mammal and bird species (Monfort 2003). Feces are generally easy to collect under field conditions, which is one of the main reasons that this approach has become so popular over the past decade. Furthermore, new scat detection dog methods increase the likelihood of locating feces in the field (see chapter 7). But feces contain large numbers of bacteria that produce enzymes that can alter the structural integrity of steroid metabolites postdefecation (Millspaugh and Washburn 2004). To minimize these postdefecation impacts, feces should be collected as soon as possible after defecation, followed immediately by treatment to minimize continued **bacterial degradation**. A disadvantage of fecal steroid monitoring, relative to urinary methods, is the need for extensive processing before immunoassay. The associated increase in time, labor, and overall cost depends, in part, on the fecal extraction method employed, including whether samples are extracted wet or dry, procedural losses are documented, and heat is used during the extraction process (Monfort 2003).

Sample Treatment and Preservation

Once collected, urine can be preserved indefinitely through frozen storage (e.g., household or propane freezer, liquid nitrogen tank) or with "field-friendly" storage methods, including absorbing urine onto filter paper (Harris and Monfort 2003) or storing it in 10% ethanol at room temperature for up to twelve weeks (Whitten et al. 1998). In contrast, if feces cannot be collected and preserved within one to two hours postdefecation, one needs to systematically evaluate the potential impact of bacterial degradation on hormone metabolite concentrations, and develop a sample storage strategy to mitigate this impact (Whitten et al. 1998; Khan et al. 2002; Terio et al. 2002; Washburn and Millspaugh 2002; Hunt and Wasser 2003; Lynch et al. 2003; Millspaugh et al. 2003; Beehner and Whitten 2004; Galama et al. 2004; Palme 2005; Palme et al. 2005). This effect must be further evaluated if the samples are treated to kill potential pathogens, as required by the US Department of Agriculture. For instance, Millspaugh et al. (2003) assessed the effect of chemical and heat treatment on glucocorticoid concentrations from fresh and frozen white-tailed deer (*Odocoileus virginianus*) and elk fecal samples stored for six days. These researchers found that fecal glucocorticoid concentrations were significantly altered by the chemical and heat treatments, although treatment in a 2% acetic acid solution followed by freezing had the least impact on the sample.

Frozen storage is generally considered the gold standard of fecal preservation methods (Hunt and Wasser 2003), but fecal steroid metabolite concentrations may vary over time even in frozen specimens (Khan et al. 2002). Adopting standardized collection and storage methods is a crucial consideration when designing any field study given that inappropriate sample storage can invalidate hormone measures. In general, it is prudent to collect feces as soon after defecation as possible (i.e., again, within one to two hours), and to maintain the specimens in a portable insulated container cooled with frozen ice packs during transfer to the field station, where samples can be frozen, treated, or processed in preparation for analysis. Remote field sites without freezers present a special challenge. In such situations, prospective research is essential to validate alternative sample preservation methods such as drying in portable ovens (Stoops et al. 1999; Lynch et al. 2003; Galama et al. 2004; Pettitt et al. 2007) or fixing samples in alcohol or other chemical-bactericidal media (Wasser et al. 1994; Khan et al. 2002; Millspaugh et al. 2003). Alternately, other field friendly extraction methods (Whitten et al. 1998; Beehner and Whitten 2004) may be available.

Simply put, investigators should presume that each species is potentially unique with respect to how reproductive and adrenal steroid hormones are metabolized, excreted, and degraded postdefecation. Descriptions of the myriad tests or experimental designs that might be employed to validate fecal sampling and storage procedures is beyond the scope of this chapter, but the burden is squarely on the investigator to demonstrate that sampling regimens employed—including postdefecation metabolization—have been tested to ensure the physiological validity of the resulting endocrine data. Endocrine data derived without controlling for the elapsed time from defecation to fecal storage, and for which the impact of storage method and the duration of fecal sample storage on hormonal measures has not been tested, should be interpreted with extreme caution. A useful option for validating fecal sample collection and storage methods is to use freshly collected feces from captive subjects maintained in zoos or wildlife centers to conduct controlled experiments.

For international field studies, specimen exportation can be avoided completely if sample processing and immunoassays are conducted in the countries where specimens are collected. This is now feasible given the advent of nonradiometric immunoassays, and even noninstrumented immunoassays (Kirkpatrick et al. 1993), which are increasingly portable and transferable to remote field sites.

Pitfalls, Cautions, and Future Perspectives

As Cervantes said, "A word to the wise is enough." This applies to ensuring that the necessary validations are conducted for each hormone assay and species of interest. Failure to do so can completely invalidate the usefulness of endocrine measures. For example, accurate pregnancy diagnosis requires that steroid measures be initially confirmed independently using alternate methods (e.g., rectal palpation, ultrasonography, pregnancy-specific proteins), as well as direct visual confirmation of neonate status. Additionally, a priori knowledge of normal endocrine excretion dynamics is essential for determining the optimal time for sampling (i.e., early- versus mid- or late-pregnancy), and the degree of individual, seasonal, and age-related effects on fecal hormone production.

Likewise, hormone tests of adrenal status require that one demonstrate a cause-and-effect relationship between a stressor and its ability to induce an associated temporal increase in excreted GC concentrations (Wasser et al. 2000; Millspaugh et al. 2003). Special caution is necessary for assessing GCs in the context of other potentially relevant biological factors, such as seasonal and diurnal rhythms; body condition; nutritional status; and the social or reproductive status, age, and sex of the animal being sampled (von der Ohe and Servheen 2002; Millspaugh and Washburn 2004; Palme 2005; Touma and Palme 2005). Further, treatment effects (e.g., human-induced stress) must be considered against the background variation from these potentially confounding factors to demonstrate that measured endocrine changes provide physiologically relevant information.

Wildlife biologists should be aware that no two immunoassays are created equal: each employs a unique antibody with a characteristic specificity or ability to recognize minute, three-dimensional structural differences among similar classes of hormones. Thus, two different assays for the same hormone (e.g., progesterone)—sold by different manufacturers or developed by separate labs—may not necessarily recognize the same exact hormone metabolite. This is because downstream hormone metabolites in excreta are diverse, and each antibody may recognize one or more metabolites that are unique for that particular assay. Results from two different labs and/or assays for the same hormone may therefore not be directly comparable, which reinforces the need for the validations emphasized above. Additionally, the storage or processing method used (e.g., ethanol boiling versus cold buffer solubilization for feces) may affect the diversity and overall concentration of metabolites quantified by any particular assay. In short, it is essential to carefully evaluate the endocrine methods employed, even when using extraction procedures or immunoassays that have been previously reported to be effective for documenting steroid metabolites in a closely related species.

Urinary and fecal steroid monitoring have now been employed in dozens of species of mammals (Monfort 2003; Palme et al. 2005) and birds (Goymann 2005; Touma and Palme 2005), although fewer studies have been conducted under field conditions (Monfort 2003). Despite progress, we still know strikingly little about the reproductive biology and stress physiology of most species of mammals and birds. Excellent new examples of field applications are being published each year, reflecting this rapidly emerging field of investigation. It is becoming increasingly common for field investigations to merge behavioral, genetic, and hormone measures to define life history requirements of individuals, populations, and species, as well as complex ecological relationships and the evolution of mating systems—including phenomena such as dominance, social stress, and reproductive suppression. Such methods are a boon to enhancing our fundamental physiological knowledge of reproductive status, health, and the effect of human disturbance on animal well-being. Increasingly, these methods are proving helpful to wildlife managers and decision makers in their attempts to ensure the survival of viable populations of carnivores and other species.

Synergy of Genetic and Endocrine Data

Advances in both molecular biology and endocrinology, coupled with noninvasive sampling, are providing new insights into the population dynamics of many species. From a single fecal sample we can now identify not only the species, but the individual and its sex, population of origin, reproductive status, and social status by amalgamating molecular and endocrine approaches. Yet, to obtain this synergy, appropriate pilot work must be conducted and samples must be treated in a manner acceptable to both disciplines.

Pilot molecular ecology work will provide information about the power of the molecular markers being used, the efficacy of the storage and extraction techniques, and the success rate of sampling from the specific species and location. Given species- and location-specific variation, results of pilot work should then guide future sampling efforts. In the field of endocrinology, pilot work is critical to providing baseline data and to ensuring that changes in assayed hormone levels are biologically meaningful and provide reliable and consistent estimates of hormone production. Such validation is often achieved by independently confirming that physiological and behavioral changes induce predictable changes in steroid levels. In addition, pilot work can determine the optimal timing for sampling, and the degree of individual, seasonal, and age-related effects on hormone production.

If one hopes to obtain both DNA-based (e.g., species, individual identification, sex, population genetic) and endocrine-based (e.g., social, physiological, reproductive status) information from the same sample, a priori planning is absolutely critical. Sampling hair, while effective for molecular genetics, is not as useful for endocrinology. Urine has proven effective and inexpensive for endocrine studies and can provide molecular genetic information as well—but probably at a diminished success rate when compared to other source material. The cost of failed DNA samples must be balanced against the savings generated in the endocrinology laboratory. Fecal samples may be the best medium for obtaining both DNA and adrenal or gonadal steroids. Freezing samples is ideal for endocrine work and is adequate for many DNA studies (although this approach never ranked highest in table 9.3 and is known to present difficulties for obtaining sufficient DNA from some species). If only one sample can be acquired, freezing should enable it to provide maximal information. Alternatively, we recommend dividing a freshly collected fecal sample into two subsamples for best results. The first subsample should be immediately frozen and sent to the endocrine lab, and the second freeze dried or placed in silica desiccant, buffer, or ethanol (depending on results from preliminary studies) and sent to the DNA lab. The use of a barcode system with multiple labels can help researchers track identical samples shipped to different labs (see box 9.3 and figure 6.16 for more details).

Given pilot studies and careful sample collection, treatment, and storage, there is much information to be gained from noninvasively collected samples toward the study of carnivore abundance, reproductive and social status, occupancy, and geographic range. Furthermore, as both molecular genetics and endocrinology are rapidly advancing fields, we expect to see new developments that will allow even more information to be obtained from noninvasive sampling.

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