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Health assessment and epidemiology

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4.1 Introduction

The interests of primatologists, veterinarians, immunologists, and disease ecologists, when taken together, create a multidisciplinary approach for identifying the ecological, physiological, and behavioral determinants of infectious diseases in wild non-human primate populations. The fruits of these combined interests are a number of published field studies of intestinal parasites and other pathogens in wild primates ("primates" to be used in lieu of "non-human primates" for the remainder of this chapter). The variety of methods employed in these studies continues to grow. Our goal here is *not* to provide a comprehensive list of protocols for sample collection and analysis, (see Gillespie 2006; Gillespie *et al.* 2008; Greiner and McIntosh 2009; Leendertz *et al.* 2006; Unwin *et al.* 2011). With this chapter, we rather outline the importance of health assessment in wild primates, suggest how this information may be used for research and conservation purposes, and focus on topics that have been stressed less in previous publications. For the novice, this discussion may help you choose appropriate methods; for the expert, this discussion may help to expand your existing toolkit or point your research in a new direction by providing additional options for consideration.

4.2 Primate zoonoses and anthropozoonoses

The following material describes health assessment as it primarily relates to infectious organisms. This of course does not negate the importance of assessment of injury and chronic conditions, although they are beyond the scope of this paper. Infectious organisms include viruses (and bacteriophages), bacteria (including rickettsiae), parasitic protozoa and helminthes (nematodes, cestodes, and trematodes), and fungi. These parasitic organisms live all or part of their lives in or on a host from which biological necessities are derived. This state of metabolic

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dependence usually results in host energy loss, lowered survival, and reduced reproductive potential. Disease or illness is the impairment of host body function due to a pathogen.

There is remarkable variation in the transmission dynamics of infectious (communicable) organisms (Anderson and May 1992; Combes 2004; Poulin 2006). The primary infection transmission routes for wild animals include fecal–oral (ingestion of contaminated food, water, or other objects), respiratory, vector-borne (e.g., mosquitoes, ticks, flies, etc.), blood-borne, sexually-transmitted, and congenital. Zoonotic pathogens are those transmitted from non-human animals to humans; anthropozoonoses are those pathogens transmitted from humans to non-human animals. Primates can serve as the reservoir host for a number of zoonotic diseases (Chomel *et al.* 2007). Given that over half of all human infections are zoonotic in origin (Cleaveland *et al.* 2001; Woolhouse and Gaunt 2007), it is important to monitor the health of wild primate populations for our own health purposes.

For example, the current HIV pandemic, with an estimated global prevalence of 33 million people, appears to have originated from non-human primate simian immunodeficiency viruses (SIV). Through the hunting and butchering of chimpanzees (*Pan troglodytes troglodytes*), western lowland gorillas (*Gorilla gorilla gorilla*), and sooty mangabeys (*Cercocebus atys*) in West Africa (Gao *et al.* 1999; Santiago *et al.* 2005; Van Heuverswyn *et al.* 2006), SIV likely entered into the human population several times and became established as HIV around 1900 AD (Worobey *et al.* 2008).

Primates are hosts for a number of malaria species (Prugnolle *et al.* 2010), and likely serve as a reservoir for some human infection, particularly from *Plasmodium knowlesi* (Cox-Singh *et al.* 2008). Malaria is a mosquito-borne disease caused by protozoa of the genus *Plasmodium* (phylum Apicomplexa, suborder Haemosporidiidea, family Plasmodiidae), with 172 named species that parasitize reptiles, birds, and mammals (Garnham 1966; Coatney *et al.* 1971).

Some pathogens transmitted to humans via contact with wild primates can be particularly deadly. For example, Cercopithecine herpesvirus 1 (B virus) is common in wild and captive macaques (*Macaca* sp.). When actively shed from the macaque mucosal epithelia, transmission to humans can, unlike infection in the reservoir hosts, result in fatal encephalopathy (Huff and Barry 2003). Nearly all cases of Ebola in humans can be traced back to the handling or consumption of infected wildlife carcasses, particularly those of apes (Leroy *et al.* 2004). *Ebolavirus* infection has wiped out several non-human primate populations, particularly great apes in Gabon, Cameroon, and Democratic Republic of Congo over the past 20 years (Leroy *et al.* 2004).

Primates can also be highly susceptible to human pathogens (Kaur and Singh 2009), due in part to phylogenetic relatedness between human and non-human primates. Wild populations are usually immunologically naïve to human pathogens, and ape populations in particular can be quickly decimated because of slow reproductive rates. For example, outbreaks of polio, measles, anthrax, and respiratory pathogens have been responsible for major mortality in wild ape populations (Leendertz *et al.* 2006). Contact between human and wild primate populations is the likely cause of several of these epidemics, particularly that of respiratory syncytial virus and metapneumovirus in chimpanzees in Côte d'Ivoire (Köndgen *et al.* 2008), as well as intestinal pathogens *Giardia* and *E. coli* in mountain gorillas and chimpanzees in western Uganda (Graczyk *et al.* 2002; Goldberg *et al.* 2007; Rwego *et al.* 2008). Primates can be very susceptible to *Mycobacterium tuberculosis*, *M. bovis*, *M. avium*, and other bacteria (Burgos-Rodriquez 2011). Monitoring the health of primate populations is obviously important for conservation purposes and for human health concerns. But these wild populations can also offer unique opportunities for addressing interesting questions about the influence of host and ecological conditions on susceptibility to infection and complex transmission dynamics. In this case, a variety of epidemiological methods can be employed.

4.3 Methods for collection and analyses

4.3.1 Study design

Several introductory texts on infectious disease epidemiology are available: Friis (2009), Heymann (2008), Merrill (2009), and Rothman, K. J. *et al.* (2009). Epidemiology is the study of the distribution (frequency, time, hosts, locations, etc.), determinants (biological, behavioral, ecological, etc.), course (etiology, spread throughout a population, effects of control measures and interventions, etc.) and outcome (morbidity and mortality) of disease in a population. There are a number of epidemiological study designs, and the most common ones used to document primate health are observational, descriptive, cross-sectional ones. These "ecological" (or correlational) studies measure exposure and effect simultaneously, and document occurrence of disease by analyzing groups of hosts in different places or in a time series. This simple study design can lead to difficult interpretation of results (as there are many potential explanations for findings). And of course these studies are subject to bias (the "ecological fallacy") because associations observed between variables at the group level may not exist at the individual level (Freedman 1999). Studies of primate disease ecology should attempt to utilize more sophisticated epidemiological designs such as case-control analysis, in which

the occurrence of disease is monitored longitudinally over time in exposed versus unexposed groups. Cohort studies could measure the same disease-free animals multiple times over a given period of time to observe development of disease in different groups. Relative risk of infection can be calculated by comparing rates of occurrence in exposed versus unexposed groups in cohort studies. But remember, the two groups ideally need to be comparable in all other ways.

Regardless of the study design, basic projects in primate disease ecology will begin with describing the *prevalence* of infection, which is the frequency of existing cases of disease in that population. These values are obviously influenced by a number of variables, including duration of disease, diagnostic abilities, and migration of hosts in and out of the population (so narrowly define your population at risk to the greatest extent possible). *Incidence* represents the number of new cases of disease in a specified population during a given period of time, which can be calculated if the host population is sampled multiple times across a given period of time. Finally, *richness* represents the number of unique pathogenic species that infects a given host at a given period of time. Richness is a measure typically reported in studies of primate disease ecology.

4.3.2 Permits

Researchers are usually required to obtain several different permits for animal sampling and specimen transport. Permit requirements vary by host and home countries and species of animal. Researchers must inquire about their specific requirements. Some more common issues include:

1. *Local (host country) permissions*: In most cases, foreign researchers must identify local counterparts and submit applications for research permits (also see Chapters 3 and 8). The local collaborators may also provide researchers with important letters of support, such as letters to customs officials describing how the researcher is traveling with necessary research supplies. Likewise, they might supply letters to airlines and shipping companies describing the context upon which the samples were collected, and confirming that they are diagnostic specimens for research purposes only.
2. *Ethical permissions*: Primate research most often requires adherence to institutional guidelines, like those imposed by research/educational organizations. In the US, these policies are usually enforced by local Institutional Animal Care and Use Committees, under the guidance of the US National Institutes of Health, Office of Laboratory Animal Welfare. Similar policies are enforced by the Academy of Medical Sciences, Royal Society, Medical Research Council and Wellcome Trust in the UK (Weatherall 2006).

Permits to ensure adherence to these guidelines are often required for release of funds from granting agencies.

3. *Exportation/importation of specimens*: Appendix I (dated 27 April 2011) of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) lists 55 primate genera or species as endangered, and therefore requires both an export permit from the host country and an import permit from the final repository of samples. These import permits for the US are granted by the US Fish and Wildlife Service, International Affairs, Division of Management Authority. For the export permit, it is necessary to identify a CITES management authority (this is different from the scientific authority) officer in the host country and supply this person with information regarding species utilized, numbers and volumes of samples, and intended uses.

For any samples that may contain etiologic agents (including vectors of disease), a CDC Etiologic Agent Import Permit is required in the US. Currently, the US Fish and Wildlife Service does not require a permit to import animal feces, as they consider feces a byproduct, not a product or derivative. Similarly, the US Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, National Center for Import and Export does not require an import permit from non-human primate samples as long as they do not contain any potential or actual zoonotic pathogens. Therefore, samples that may contain such pathogens (including those discussed throughout this chapter) must be rendered inactivated, as through the use of preservatives. If the researcher is specifically diagnosing a biological agent determined to have the potential to pose a very severe threat to human or non-human animal health, they may also have to conform to Select Agent regulations in the US. Importation of soil (i.e., for microbial analysis) into the US is regulated by the US Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine program, and usually requires a permit.

When permits are not required for various reasons, it is still a good idea to obtain letters from the above-mentioned organizations stating that permits are not required. Permits and letters from the various organizations should be affixed to both the outside and inside of shipping containers.

4.3.3 Biosafety

Specimens from primates must be handled carefully. Researchers should be trained in both biological and chemical safety, and follow good general laboratory practices even in the field. While most investigators are careful to utilize personal protective

equipment in the laboratory when working with primates (Morton *et al.* 2008), fewer are inclined to do so in the field. Exceptions, of course, include long-running projects like the Mountain Gorilla Veterinary Project that regularly monitors the health of their researchers (The Mountain Gorilla Veterinary Project Employee Health Group 2004).

Institutional Biosafety Committees may require applications for research involving animal handling or collection of biological specimens in general. Many of these regulations are set forth by the US Centers for Disease Control and Prevention's Office of Safety, Health and Environment and the World Health Organization's Collaborating Centre for Applied Biosafety Programmes and Training.

4.3.4 Sample collection

The pathogens or other measures of health you choose will be determined by your research question, which should be influenced by what you know about the natural history of your primate host. However, field conditions will determine the types of specimens you can collect.

4.3.4.1 Non-invasive samples

At a minimum, clinical signs like decreased activity, visible weight loss, overt injury, hair loss, and loss of appetite can be recorded. Feces, collected immediately following defecation, can be sealed in polypropylene tubes, kept on ice, and frozen back at camp. Fecal samples should be macroscopically inspected immediately, and consistency, the presence of blood, and the presence of cestodes or nematodes should be noted. Samples should be free of water, urine, soil, or other contaminants, and so should be collected from the center of the fecal mass. Feces can be mixed with RNAlater® for genetic analyses, or even desiccated for later hormone analyses (Muehlenbein 2006; Muehlenbein 2009). Fecal samples can be preserved in 10% neutral buffered formalin, polyvinyl alcohol, Schaudinn's fixative or SAF fixative for later parasitological analyses, or analyzed immediately using a direct wet smear to identify motile protozoa. Samples can also be placed in 10% glycerine or various transport media over the short term for bacterial culture. Several companies (e.g., Meridian Biosciences) provide excellent collection materials with reagents pre-aliquoted. Samples are usually preserved using a ratio of three parts preservative to one part feces (approximately 2 grams of feces). Tubes should be thoroughly labeled, sealed with ParaFilm, shaken to mix the contents well, and kept in a cool dry place until analysis.

Urine can be frozen, mixed with RNAlater®, and even spotted and dried onto filter paper (Simon & Schuster #903). Other non-invasively collected samples include saliva from food wadges, sperm plugs, and even blood found on foliage.

Ticks, fleas, mites, lice, mosquitoes, and leeches can be collected from nests or from the surrounding area using nets, suction devices, traps, combs, sheets, and sifters. Excellent resources on specific methodology can be obtained from various agencies such as the US Department of Agriculture, Agricultural Research Service, Insect and Mite Identification Service. Soil sampling for microbial identification is also described in detail by the US Department of Agriculture's National Soil Survey Center and other resources (Carter and Gregorich 2008).

4.3.4.2 Invasive samples

If it is feasible to anesthetize the animals, several samples should be taken. You can record weight and body temperature and note the presence of any injuries. Blood should be collected from the femoral, tibial, or brachial veins using a sterile technique and materials (like BD disposable needle sets and EDTA Vacutainer tubes). Note that there are limits on how much blood can be collected from an animal at any one time, and this is based on weight of the animal, the frequency of sampling, and your own Institutional Animal Care and Use Committee's regulations.

Blood can be frozen whole, mixed with RNAlater® (500 µl blood to 1.3 ml RNAlater®), or spotted and dried onto filter paper (Simon & Schuster #903). Serum can be separated via centrifugation and frozen. Thick and thin blood smears (films) should be made following techniques described in Houwen (2000). Blood for smears and spots can also be collected via finger or ear stick. Saliva can be collected and mixed with Qiagen's RNAprotect® saliva reagent (150 µl saliva to 1 ml reagent). Swabs can be desiccated or placed in a variety of bacteria or virus culture media. Tissues from necropsies (Travis 2009) can be preserved in ethanol, RNAlater® or 10% formalin. Ectoparasites can be collected from the host, killed using various liquid and solid substances inside killing jars, and preserved via freezing, desiccation, ethanol, or RNAlater®. In all cases, tubes and other containers should be properly labeled using a solvent-proof pen. The more analyses you want to make, the more sample aliquots you should take.

4.3.4.3 Transportation

Airlines have strict restrictions regarding liquids, flammables, and dangerous substances. Whereas some of us remember bringing blood samples back from the field in a cooler in the overhead compartment of an airplane, we are now largely restricted to shipping samples back or placing them in checked luggage (although proper packaging must be utilized). Dry ice and liquid nitrogen are usually not allowed on airplanes. Acceptable products include industrial-strength freezer packs (for −20 °C) and "dry shippers" which contain an absorbent material for keeping

samples at cryogenic temperatures (−150 °C). Researchers must bring these things with them, or have them available in the country (including liquid nitrogen for dry shippers). In any case, packaging conditions are outlined by the International Air Transport Association, and require appropriate primary and secondary containers and labels.

4.3.5 Sample analyses

Methodological standardization is necessary if results from different studies are to be even remotely comparable (Muehlenbein 2005). That said, comparisons will always be limited by an enormous range of inter-observer error when more than one laboratory is involved in the diagnostics.

4.3.5.1 Feces

If you have decided to analyze the fecal material for parasites yourself (but See Section 4.4), Ash and Orihel (1991) and Garcia (2007) are invaluable references. Proper identification of intestinal parasites usually requires the combination of different concentration techniques. Sedimentation via centrifugation helps to recover all oocysts, eggs, and larvae, and flotation procedures using high-specific gravity liquids allow the separation and detection of all but the heaviest larvae and eggs. The most common sedimentation techniques utilize ethyl acetate or ether, and work via centrifugation to extract fat and debris from the stool and concentrate the parasites in a lower sediment suspension. The most common flotation techniques utilize zinc sulfate, sugar, or sodium nitrate to cause lighter parasites to rise to the surface while debris and heavier parasites sink. Although others have suggested that zinc sulfate flotation and formal-ether concentration methods are inadequate for detecting primate parasites (Gillespie 2006; Gillespie *et al.* 2008), these methods have been used extensively in veterinary medicine. Furthermore, there has been no adequate comparison of these techniques using primate samples.

Examination of a permanent stained smear via oil immersion will also aid in the correct identification of a variety of intestinal protozoa. Examination of a permanent stained smear can be performed when it is convenient for the examiner, and the smears represent a permanent record of the parasite. Depending on the difficulty of the preparation and the amount of time available for staining, the examiner has a wide variety of staining techniques to choose from, including the trichrome stain, iron hematoxylin stain, and acid-fast stains. However, before the permanent stains can be performed, the smears must be prepared depending on how the fecal sample was preserved.

Different techniques can be used for quantification of helminth eggs including the Kato thick smear and the Stoll technique. However, correlating egg counts

with the predicted number of parasites present is not very effective. Estimates of worm burdens are highly vulnerable to variability in fiber content, consistency, parasite factors, and host characteristics (Stear *et al.* 1995). Additionally, egg production by parasites also varies with the age of the parasite population, the presence of co-infections by other parasites, and the level of the parasite population.

Nematode infections may be diagnosed via specific culturing techniques, known as coproculture, that concentrate larvae that typically hatch in soil or tissue. Culturing of feces allows for the detection of larvae in feces when they cannot be detected via concentration methods or distinguished from other first-stage larvae. Culturing also allows for the development of filariform larvae, which aids in the differentiation process of diagnosing species (Garcia 2007). Common techniques include Harada–Mori filter paper strip culture and the Baermann procedure.

Whichever combination of methods you choose, the entire area of the coverslip on the slide should be examined with a low-intensity light and a low-power objective (10x). A higher objective (40x) should be used for identifying specimens. You should measure the size of the parasites using a calibrated ocular micrometer, and photograph questionable specimens if possible. Iodine or buffered methylene blue can be used to stain the specimens.

In addition to classical parasitology, other techniques like immunofluorescence (Kowalewski *et al.* 2011), pyrosequencing (Yildirim *et al.* 2010), and RT-PCR (Johnston *et al.* 2010) can now be employed on feces. These tests are more accurate and rapid, but also much more expensive. Use of these and other techniques have facilitated the identification of SIV (Santiago *et al.* 2003), malaria (Prugnolle *et al.* 2010), and metapneumovirus (Kaur *et al.* 2008) from primate fecal samples.

For bacteria, ideally, samples should be cultured immediately after collection on media appropriate for detecting the microbe of interest. This can be done in the field if refrigeration and incubation are available. Various culturing techniques are described in the US Food and Drug Administration's Bacteriological Analytical Manual, available free online. The shelf life of samples can be extended through the use of an appropriate transport medium, although such media only preserve the infectivity of bacterial cells for time periods on the order of days to a week. Freezing samples for future analysis is possible, but this likely reduces infectivity, even in the presence of such preservatives as glycerol. In situations where infectivity cannot be preserved, molecular detection methods can be used. These are highly sensitive but preclude further analysis of the biological properties of cultured organisms. Techniques include multilocus sequence typing, and restriction fragment length polymorphism analysis of PCR-amplified genes encoding 16S rRNA, allowing

researchers to characterize the epidemiological relationships between pathogens (Goldberg 2003; Goldberg *et al.* 2006).

4.3.5.2 Blood

Thick and thin blood smears (films), usually stained with Giemsa, are helpful in diagnosing protozoan parasites, like malarias, babesias, and trypanosomes, based on morphologic features. A minimum of 200 microscopic fields of the thin smear (at 100x magnification) and 100 fields of the thick smear should be evaluated. If blood parasites are suspected but still not found in the thick or thin films, blood samples can be concentrated using several techniques such as Knott concentration, membrane filtration, and density gradient centrifugation.

Hematocrit, packed cell volume, mean corpuscle volume, hemoglobin, and erythrocyte sedimentation rate can all be determined using field-friendly supplies. Red blood cells can be lysed, the peripheral blood mononuclear cells separated and fixed in formaldehyde, and a white blood cell differential performed (much more accurately than via microscopy) using flow cytometry, but samples must be analyzed within a very short timeframe of days. However, physiological ranges for normal blood parameters in many primate species are available through the International Species Information System.

Antibodies against specific infections can be determined in whole blood and serum using enzyme immunoassay and western blots (Goldberg *et al.* 2009; Khan *et al.* 2006). Blood samples can be used on microarrays or "virus chips" (e.g., Greene chip) as well as with mass spectrometry (Abbott's PLEX-ID and other systems by AnagnosTec and bioMerieux) for casting a wide diagnostic net.

PCR has the ability to detect very low levels of parasitemia. Additionally, PCR represents a diagnostic test that can identify parasites to the species level, even in mixed infections. This is particularly the case with primate malarias. Whereas a good morphological characterization is always desirable, malarial parasites are identified using few morphological characteristics, and even experts must take the host into consideration as a context to interpret such traits. The gene encoding the mitochondrial Cytochrome b (cytb) is now frequently sequenced for identification of closely related malaria species (Escalante *et al.* 1998).

PCR inhibitors, genotypic variants, and DNA degradation can affect PCR results (see Chapter 14 for more details). As PCR techniques become more utilized in the future, researchers must keep in mind the sensitivity of PCR detection, which can be influenced by the collection mode and storage conditions of blood samples. These factors are especially important in cases of low parasitemia, mixed infections, and when comparing data from multiple laboratory and field settings

(Farnert *et al.* 1999). Avoiding sample contamination is another reason to wear personal protective equipment when collecting samples.

4.3.5.3 Rapid tests

There are a number of field-friendly rapid tests for parasites, bacteria, and other indicators of infection status. These include urinary dipsticks (e.g., Bayer Multistix for assessment of leucocytes, ketones, glucose, and other factors in urine), rapid culture supplies for coliform analysis of water (e.g., ColiComplete from BioControl Systems and ColiScreen from Hardy Diagnostics), and various lateral flow immune-chromatographic antigen-detection tests (e.g., PrimaTB STAT-PAK assay from ChemBio Diagnostic Systems, VetScan Giardia rapid test from Abaxis, ImmunoCard Stat! products from Meridian Bioscience, and the Malaria Pan/Pv/Pf test by Core Diagnostics). Before employing these tests, it is important to consider whether they can in fact detect your organism of interest (i.e., they may not detect the species of parasite that is commonly found in your host population), and the sensitivity of the test, as many of these tests may have unacceptably high rates of producing false negatives.

4.4 Some modest advice

Experience allows us the luxury of hindsight. And many publications in primate disease ecology suffer from a number of methodological flaws. Here we offer some modest advice we hope will be helpful, so that someone might also learn from our mistakes.

1. *Find an expert*: Just as tests must be reliable (precise, repeatable), valid (accurate; measures what it is intended to measure), sensitive (ability to correctly identify those with disease), and specific (ability to correctly identify those who are healthy), those conducting the analyses must too be proven. Measurement error is caused not only by the use of inappropriate techniques, but also by untrained individuals. No-one is qualified to conduct the majority of the tests described above just because they have read a book on parasitology, or have taken a course in parasitology. New researchers should find an expert with an advanced degree in tropical medicine if they are going to use parasitological techniques.
2. *Involve local colleagues beyond sample collection*: Local colleagues would benefit from being trained in whatever techniques they may be able to perform in the host country. Not all samples need always be exported from a host country to a foreign lab; in many cases, local laboratories exist or can be

developed (Cheesbrough 2005). Contributing to the education and economic development of our host countries is vital to sustained collaborations.

3. *Choose the research questions before the research methods*: Too many students become enamored with some romantic topics like parasitology or endocrinology. They decide they want to use these methods, sometimes for the wrong reasons. They then work backwards to identify a research question so that they can use these methods. In most of these cases, the research questions turn out to be mediocre. Choose an important question, generate a list of predictions and potential confounding variables, and then seek advice about the best methods to evaluate these questions. It may be the case that you should be using a measure of animal health rather than infection status (as the two are not the same). Additionally, conducting a pilot project will minimize the potential for serious problems with techniques after the data are collected.
4. *Do not collect samples randomly from unknown individuals*: This will produce an inaccurate measure of prevalence by basing analyses on parasites per sample instead of parasites per animal. Huffman *et al.* (1997) demonstrated that significant reporting bias is created when one uses the number of samples instead of the number of individuals to calculate the prevalence of parasitic infection in a population. Furthermore, fecal samples are not randomly distributed in the environment, as ill individuals defecate more frequently than healthy hosts. Random collection of feces would then result in sampling bias (i.e., some animals sampled more than others). This is particularly problematic if your pathogen of interest is not evenly distributed throughout the host population, which is usually the case. Samples should be collected from known animals immediately following defecation, and prevalence estimates should be based on number of positive animals, not number of positive samples. It is possible to genotype the samples in order to determine sample identity, although this is an extremely expensive option.
5. *Multiple samples should be collected from each animal across time*: Intestinal parasites are shed intermittently. Therefore a minimum of three samples, collected on non-consecutive days, is necessary for accurate diagnosis of intestinal infection. For example, Muehlenbein (2005) demonstrated that not one of the twelve parasitic species recovered from the chimpanzee Ngogo group in Kibale National Park was found in *all* samples from any one animal, and the most commonly occurring parasites were found in all of the serial samples of only a fraction of the chimpanzees sampled. Cumulative parasitic species richness for the chimpanzee hosts significantly increased for every sequential sample (up to four samples) taken per animal during this study.

While a single sample may be adequate for determining antibody levels indicative of past infection, current infection usually requires multiple samples.

6. *Try to sample as much of the population as possible*: Sampling error can happen when only one group of animals is sampled, and if that group of animals is not representative of the entire population (perhaps because they are better habituated than others). Furthermore, sample size must be large enough to have statistical power to detect important differences. We recommend using the free software G*Power (Erdfelder *et al.* 1996). Only a handful of published studies on primate disease ecology have ever reported results of their sample size or power analyses.
7. *Try to sample across seasons*: There is likely seasonal variation in pathogen transmission in wild primates (Gillespie *et al.* 2010; Huffman *et al.* 1997). And many factors such as rainfall, temperature, sex, age, reproductive state, body condition, dominance rank, host population density, and daily range could influence these transmission dynamics. It is important to try to account for as many of the variables that may influence susceptibility to pathogens as possible as well as contact with them.
8. *Be conservative in your interpretations*: It is difficult to determine whether observed associations are causal, as the guidelines for judging causation are difficult to establish in wild primate studies. Among other considerations for causation, the cause must precede the effect, there must be a strong relationship between the cause and effect (the cause should covary with the effect), the results must be consistent with other studies, and the mechanism of action must be plausible (Rothman 1988). In field studies involving primates, it is usually impossible to demonstrate that increased exposure is associated with increased effect, nor to illustrate that removal of the cause leads to reduction of disease risk. There can be much confounding (when another factor is associated with both exposure and disease, and it is not accounted for in analyses and hypothesis generation) in ecological studies guided by inductive reasoning.
9. *Realize that not all infections are virulent in primates*: We need to know how naturally occurring infections impact the survivorship and reproduction of wild primates. Morbidity and mortality is to be expected under some, but not all, conditions. Some animals may be resistant to infection, while most may develop a tolerance for chronic infection. Many infections are thought to impair nutrient absorption, cause anemia, and influence energy expenditure. This is certainly the case for some infections like *Oesophagostomum*. Low levels of chronic infection matched with a relative absence of clinical

signs most likely indicate that many infections are well tolerated. This is probably the case for most intestinal parasites of wild primates. These infections may only cause overt disease under certain circumstances, such as following seasonal fluctuations in food or during breeding seasons. Too many recent publications assume that these intestinal infections cause high levels of morbidity, when in fact there is little evidence for this assumption at this time. Research should be focused on determining the effects of parasitism on primate host morbidity and mortality. Research should also be focusing more on pathogenic viral and bacterial infection rather than basic intestinal parasitism, although the latter will continue to be important.

10. *Consider measuring immune function*: Nearly all measures of primate health have been limited to determination of exposure (i.e., baseline parasite levels). It is feasible under certain circumstances (usually involving collection of invasive samples) to go beyond reporting parasitemia and signs of illness, to include immunological measures such as immunophenotyping (e.g., white blood cell differential), lymphocyte proliferation (ability of T and B cells to undergo mitosis *in vitro* following exposure to a mitogen), hemolytic complement activity of serum, *ex vivo* bacteria killing abilities of blood and urine, antibody and cytokine levels in blood and urine via enzyme immunoassay, multiplexing, western blot, immunofluorescence, or haemagglutination. Gene expression of immune effector pathways can be determined using quantitative PCR or microarrays. Concentration and activity of complement proteins, lysozyme, antimicrobial peptides, NK cells, nitric oxide, and macrophage phagocytosis can all be determined using a variety of assays. Which assays you choose should depend on your model system and availability of blood. Many of these assays represent functional, integrated, biologically relevant measures of different immune pathways (Boughton *et al.* 2011; Demas *et al.* 2011).

But remember, baseline immune measures in wild animals do not necessarily represent a "disease absent" state, as some upregulation of immunity due to the ubiquitous presence of parasites is to be assumed. Immune responses are influenced by more than just infection, including factors such as reproductive state, energy balance, stress, and season. Furthermore, it is advised not to rely solely on a single immune measure, as this cannot adequately represent the functioning of an entire system. Multiple measures would ideally be combined with assessment of animal infection status.

4.5 Going beyond basic health monitoring

Primate epidemiology should attempt to go past basic health monitoring to answer questions regarding risk assessment (i.e., likelihood of changes in disease patterns; Travis *et al.* 2006) and most importantly the causal factors of changes in disease dynamics within and between populations.

4.5.1 Behavioral ecology and disease risk

Population density, mating system, geographic range size, body mass, terrestrial substrate use, diet, and group size are all likely related to risk of infection in complex ways in wild primates. In general, arboreal primates may have fewer parasites than terrestrial ones due to less potential contact with feces and soil containing parasites (Muehlenbein *et al.* 2003). Howler monkeys may defecate in the peripheral areas of the canopy in order to avoid contamination of food with intestinal parasites (Gilbert 1997; Henry and Winkler 2001). Nest building and alteration of sleeping sites may reduce parasite exposure (including infection from malaria via mosquitos) in primate hosts (Hausfater and Meade 1982). And chimpanzees and baboons may dig wells to specifically filter water and decrease the likelihood of water-borne infections (Galat *et al.* 2008).

Pathogens may influence primate sociality, and social behaviors can influence risk of infection (Nunn and Altizer 2006). If contact or proximity occurs frequently among individuals in dense populations and elevates the rate of disease transmission, then higher levels of gregarious behavior or sociality should result in greater infections within social systems (Altizer *et al.* 2003). Parasites may influence primates to emigrate out of a larger group in search of a smaller one, or dominant individuals may force subordinate animals to leave larger groups (Freeland 1976). Furthermore, if pressure from pathogens increases emigration from a primate social group, then subordinate, younger, or elderly individuals, as well as the dispersing sex, are likely to experience the most pressure (Nunn and Altizer 2004).

Primates could potentially reduce their risk of disease by avoiding group members displaying visible cues of infection, including physical, behavioral, and olfactory signs of infection. This could influence grooming patterns and distances between individuals. An increase in territoriality might result in greater use of a group's home range, which may lead to exposure (or re-infection) of pathogens (Ezenwa 2004; Stoner 1996). Despite these interesting predictions, there is little evidence to definitively support these ideas. Testing these predictions in wild primates will prove difficult, but certainly rewarding.

4.5.2 Modeling infection transmission in primate populations

Traditional models of susceptible, infected, and recovered hosts in a fixed population underestimate the complexity of host ecology, transmission dynamics, pathogen virulence, spread of resistance/tolerance, and much more (Brauer and Castillo-Chavez 2001). In addition to detailed comparative analyses (see Nunn and Altizer 2006), agent-based modeling is being more frequently used to model these complex ecological systems (Nunn 2009). These models attempt to account for spatial structure as well as interaction patterns of individuals (also see Chapter 9). For example, dominance rank may influence the likelihood of infection transmission due to variation in diet, contact between animals, and physiological stress (Muehlenbein and Watts 2010).

A variety of computer programs are available for agent-based modeling depending on the programmer's level of experience. Software that is most often used for the social sciences includes LSD, MAML, MAS-SOC, Repast, NetLogo, FAMOJA, SimBioSys, StarLogo, Sugarscape, and VSEit. The primary step in constructing an agent-based disease model is building the host population. Here, the researcher needs to decide group composition, size, structure, dispersal, sex, and spatial organization (Nunn 2009). Decisions need to be made regarding the number of neighbors the host population may have, how often they come into contact, and if there are any geographical or social barriers that may exist between the two. The researcher needs to consider life history variables, such as mortality: Which agents die of natural causes and how many? Which agents die from the disease? These types of questions are important, since those killed by natural causes most likely represent a population with a constant size, while populations with a large number of agents that die from disease most likely have a decreasing population size; any changes in population size can affect subsequent spread of disease as the model progresses. Additionally, the researcher needs to make decisions about which variables will fluctuate and those that will remain static, as in models demonstrating the evolution of certain traits. Taken together, these parameters provide vital clues as to how a disease might spread in real time populations.

4.5.3 Assessing the impacts of anthropogenic change on primate health

Increased contact between humans and wild primates will likely lead to changes in infection profiles in all populations concerned. Given the rapid nature of anthropogenic modifications of our physical environment, the likelihood of pathogen transmission to wild primate populations in the future is significant. Human and livestock populations continue to grow rapidly, increasing the number of hosts potentially susceptible to novel infections. Mass transportation of people,

products, livestock, and vectors of disease bring each of these closer to one another, and more quickly at that. Population movements due to war, social disruption, and rural-to-urban migration, in addition to general urbanization, increase the densities of non-immune human hosts and pose significant sanitation problems. Changes in water usage, such as during the construction of dams, culverts, and irrigation systems, can increase the potential breeding sites of vector species like mosquitoes and snails. Human encroachment into previously undisturbed areas increases remote area accessibility and introduces more vectors and reservoirs of infection to new hosts. Encroachment, extensification of agricultural land, and urban sprawl all alter population densities and distributions of wildlife, which changes disease dynamics. Forest fragmentation can produce an "edge-effect," increasing the flow of organisms across ecotones, novel species contact, and the likelihood of infection transmission between organismal populations. Biodiversity loss due to global climate change, deforestation, the spread of invasive species, overexploitation, and other causes increases the likelihood of cross-species transmission. Increased inbreeding and decreased genetic diversity in remaining wildlife populations could even facilitate further outbreaks due to impaired immune functions in host animals.

Forest fragmentation has been associated with a variety of infection patterns in wild primates (Gillespie and Chapman 2006; Gillespie and Chapman 2008; Goldberg *et al.* 2008). In these and other examples, it remains to be seen whether or not changes in infection patterns are due to alterations in host susceptibility as the result of altered distribution of nutritional resources, altered transmission dynamics (such as increased contact with humans and livestock), or some other factor. Forest habitats may differ in complex ways other than just by degree of fragmentation, including amount of human contact, population density of hosts, fruiting and flowering schedules, amount of ground water, and so on.

Recreational use of natural areas can also affect the risks of zoonotic and anthropozoonotic pathogen transmission. Habituation of animals to human presence can increase the likelihood that animals will actively seek out contact with humans, particularly in the form of crop raiding and invasion of garbage pits and latrines. Despite the fact that primate ecotourism is increasingly perceived as a venue for promoting awareness about conservation issues, tourist–wildlife contact has the potential of producing devastating health and economic outcomes (Muehlenbein and Ancrenaz 2009). Understanding the risks of pathogen transmission from tourists to wildlife is a necessary, but overlooked, aspect of wildlife conservation; information regarding risk of transmission in these different locations could help to optimize responsible ecotourism for animal well-being and economic development.

The relative contribution of tourists to the spread of pathogens to wildlife is largely unknown, but the number of tourists visiting wildlife sanctuaries worldwide is increasing dramatically. The majority of tourists who visit wildlife sanctuaries arguably underestimate their own risk of infection, as well as their potential contribution to the spread of diseases themselves. Using the largest survey ever of ecotourist health behaviors at Asia's most frequented wildlife tourism destination, the Sepilok Orangutan Rehabilitation Centre in Sabah, Malaysia, we have demonstrated relatively low levels of vaccination in visitors (Muehlenbein *et al.* 2008). Many tourists had animal contact immediately before coming to Sepilok, and many of them had at least some basic knowledge about infection transmission (i.e., medical-related occupation) (Muehlenbein *et al.* 2010). Despite their interests in environmental protection, these ecotourists very likely create unnecessary risk of infection transmission to wildlife; despite the fact that 96% of respondents believe humans can give disease to wild animals, 35% of them would still try to touch a wild monkey or ape if they had the opportunity (Muehlenbein 2010). Clearly this problem deserves more attention, specifically by comparing risk factors at primate-based ecotourism destinations that differ significantly by visitor characteristic and degree/type of human–wildlife interaction. The tools described above may help equip researchers working with wild primates to begin to address this and other related research questions, including those which can contribute immediately and directly to primate conservation.

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