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Methods for the Study of Bat Endoparasites

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THE METHODS AND TECHNIQUES presented herein are intended to encourage biologists who study bats to collect data on endoparasites. The study of the relationships among parasites of bats offers opportunities for better understanding the complex life histories of both taxa. In this chapter, we outline methods for collecting parasites from bats that maximize the amount of morphological and molecular information available from each group. We also provide a brief historical background for the study of the endoparasites of bats.

HISTORICAL BASIS OF BAT PARASITOLOGY

Helminth and protistan (protozoan) parasites that are known to occur in bats are both morphologically and phylogenetically diverse and include many species of tapeworms (cestodes), flukes (trematodes), Nemata (nematodes), eimerians (coccidia), tongue-worms (pentastomes), and flagellates (trypanosomes). Although many species of these parasites have been described and enumerated from bats, little is known of the coevolutionary, phylogenetic, or ecological relationships of parasites and their bat hosts. Despite the increased interest in ecological studies of bats (e.g., Kunz and Fenton, 2003; Zubaid et al., 2005), there has been little emphasis on the study of their endoparasites (although see Coggins, 1988). It was not until 2002 that the partial lifecycle was documented for one of the most common nematode parasites of bats in the new world, a filarioid nematode of the genus *Litomosoides* (see Bain et al., 2002).

Müller (1780) provided one of the earliest published reports of trematodes from bats, with a description of *Plagiorchis vespertilionis* (Müller, 1780) from *Vespertilio auritus* (= *Plecotus auritus*) in Europe (see Tkach et al., 2000, for details).

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Gmelin (1790) reported the first nematode (*Nematoides vespertilionis*) from *Plecotus auritus* was reported by (for additional details see Stiles and Hassall, 1920, and Stiles and Nolan, 1931), whereas the first nematode (capillariid) from a New World bat was reported by Kolenati (1856). Parona (1890) described *Neonicola novellae* (Parona, 1890), the first acanthocephalan reported from *Artibeus jamaicensis* collected in Puerto Rico. Cestodes from bats were first reported by Gmelin (1790), whereas the coccidian *Eimeria viridus* (Labbé, 1893) was first reported from bats by Labbé (1893).

We estimate that approximately 4,400 species of endoparasites will eventually be described from the approximately 1,116 known species of bats. This calculation assumes some host specificity and is based on a conservative estimate of only four species of helminths and protists per species of bat.

IMPORTANCE OF BAT PARASITOLOGY

Understanding the diversity of endoparasites in bats may lead researchers to ask different and perhaps deeper and broader questions about the ecology, systematics, biogeography, and evolution of bats as hosts than would occur to those same researchers without considering these organisms as subjects of study. Endoparasites with complex life cycles (those that use an intermediate host such as an arthropod and mammals as definitive hosts) are excellent sources of information on local and regional biological diversity (Gardner and Campbell, 1992; Brooks and Hoberg, 2000; Hoberg et al., 2003). From a zoonotic and animal health perspective, trematodes of bats have also been shown to be involved in the transmission dynamics of *Neorickettsia risticii*, which is the causative agent of Potomac Horse Fever (Pusterla et al., 2003; Gibson et al., 2005).

Although much has recently been published on the phylogenetic relationships among bats (Wang et al., 2003; Hooper et al., 2003; Porter et al., 2003; Dávalos and Jansa, 2004; Teeling et al., 2005), there has not been a corresponding increase in research on the evolutionary and phylogenetic relationships of the parasite fauna of chiropterans. This lack of research on the phylogenetic database of parasites of bats limits the kinds of studies that can be completed that require well supported phylogenies of both hosts and their parasites (Brooks and Hoberg, 2000; Brooks and McLennan, 2002).

Obtaining data pertaining to the endoparasites of bats collected in the field or in captive studies is labor intensive and time-consuming (Ubelaker, 1970; Coggins, 1988; Gardner, 1996). However, endoparasites are integral to the ecology and the study of behavior of bats and ideally should be sampled at the time hosts are collected. Recent research on the diversity of endoparasites of mammals in the Neotropics (Gardner and Duszynski, 1990; Duszynski and

Gardner, 1991; Scott and Duszynski, 1997; Duszynski, Scott, and Zhao, 1999; Brant and Gardner, 2000; Hugot and Gardner 2000; Gardner and Pérez-Ponce de León, 2002; Jiménez-Ruiz and Gardner, 2003) could not have been completed without the close collaboration of parasitologists and mammalogists who collected, processed, prepared, identified, and placed into recognized museum collections the mammals that served as hosts. The most efficient and cost-effective method of obtaining data on the endoparasite fauna of bats is to preserve the parasites when animals are being collected, processed, or studied in the field. This seems obvious, but many (perhaps most) bat researchers discard parasites out of hand either because they are unaware of how to collect them or because they feel that it takes too much time to do so. Examples of the types of data on endoparasites that could be obtained when a bat is collected or handled include prevalence, intensity of infection, and distribution (see Lotz and Font, 1985; Coggins, 1988; Lotz and Font 1991; Lotz et al., 1995). The fields of historical ecology (*sensu* Brooks, 1985), co-evolutionary and biogeographic research (see Hoberg et al., 2003), and emerging infectious diseases (Brooks and Hoberg, 2008) invite investigations of the biology and phylogeny of hosts and their parasites.

Mark-recapture studies and studies of captive animals require researchers to employ different methods to identify endoparasites than those used in standard field- or museum-based specimen collections. For example, it is not possible to conduct detailed morphological analyses or even reliable identifications to the level of the family or genus of trematodes, cestodes, or nematodes from only eggs of these helminths that may be passed in or recovered from the feces of bats (although see discussion of DNA bar coding). In contrast to helminth parasites, many species of intestinal coccidian parasites can be investigated in sufficient detail only from fecal samples taken from live (or recently euthanized) bats (Scott and Duszynski, 1997).

Studies of the systematics and ecological characteristics of bats and their parasites require proper identification of both the parasites and their hosts (Simmons and Voss, this volume). If the endoparasites associated with a bat are not collected, preserved properly, and deposited in a recognized museum, species-level diagnostic characters (i.e., morphological characters) will almost certainly be destroyed or lost. Moreover, improper preservation of endoparasites will severely limit—and most likely prevent—research based on intact DNA molecules of the specimens collected.

Recent studies on bats have linked adult endoparasites with both the transmissible form (juvenile or larval) at the morphological level with markers at the molecular level. Given the rapid advances in development of DNA-level species tagging or DNA barcodes (Marshall, 2005), appropriate data and materials (collected at the time of capture and processing of the bat and its endoparasites)

are needed for future research, especially as molecular libraries and the phylogenetic databases continue to grow.

To enable connectivity between parasite genomics and morphology, several institutions have established cryostorage facilities such as the cryogenic facilities at the Museum of Texas Tech University, the Museum of Southwestern Biology of the University of New Mexico, the Parasite Genomic Research Facility (PGRF) in the Manter Laboratory at the University of Nebraska-Lincoln (Anonymous, 2006), and the Consortium for the Barcode of Life (CBOL).

COLLECTING DATA ON ENDOPARASITES

Data should be collected in the field laboratory or bat research laboratory in assembly-line fashion, with different individual researchers assigned to different tasks. For example, if large numbers of host animals (more than 20 per day, depending on the size of the field or lab crew) are to be processed and saved as museum specimens (see Simmons and Voss, this volume), at least two people should be assigned to search for endoparasites.

Considerations for Collecting Reliable Data

It is important to note that proteolytic enzymes begin to break down helminths that live in the intestine of the host immediately after the host dies. The hooks on the scolex of a cestode can fall off within minutes (or sooner) if the specimen is not processed quickly. The rate of autolysis relative to a specimen depends on the species of host and ambient (environmental) temperature. Immediately after death of a bat, its body temperature may increase or decrease depending on field conditions. Thus, in preparing helminth parasites for study, the investigator should consider changes in the postmortem temperature of the bat. Keeping a host specimen cool may not be an option in many field situations; thus, the only way to keep specimens of parasites and their tissues containing genetic data from decomposing due to high temperatures and autolysis is to stagger the processing of the bats to ensure that the processing and preparation rate of internal organs keeps pace with the rate that host specimens are acquired, euthanized, and processed.

Before processing is started, the field collector must make important decisions relative to the material of interest (e.g., rare species of bats), preservation techniques to be used, and type of materials to be preserved for laboratory study). Field researchers should be prepared to make adjustments depending upon the successes or failures of capturing host specimens and the diversity and densities of the endoparasites encountered. This can range from many bats collected, each with only a few species and individuals of endoparasites, to few bats, each with many species and large numbers of helminths present. The latter situation (e.g., many bats with many parasites) may

create a challenge for the field team when some processing protocols force the team to focus on certain groups of parasites more than others, or when the parasites must be preserved in a particular way. Establishing standardized criteria to collect and preserve specimens of higher or lower importance is needed to ensure consistency in collection of data.

Locating Endoparasites in Bats

Endoparasites of bats may be found in many types of tissues or organs, including each part of the digestive tract (e.g., buccal cavity, esophagus, stomach, small intestine, large intestine, and rectum). Parasites can also be found in the scrotal sac, eyes, nasal cavities, peritoneal and/or pleural cavities, and other organs such as the brain, liver, or kidneys. Nematodes can be found encysted or free in the dermis, subdermis, and just below the dermis (in the muscular fascia) and within the muscles, lungs, trachea, gastrointestinal tract, urinary bladder, kidneys, liver, gall bladder, and/or other glandular organs. Adult filaroid nematodes can often be found writhing among the intestines in the peritoneal cavity as well as around the lungs and heart in the pleural cavity. Some nematodes live in subcutaneous cysts in the membranes of the wing, feet, and uropatagium. Juvenile nematodes of the Filarioidea are often found in blood or lymph where they can be ingested during a blood meal by the arthropod intermediate host (flies [Diptera], mites and ticks [Acari], or fleas [Siphonaptera]).

Cestodes, or tapeworms, are usually found in the small intestine of bats. Digenetic trematodes are commonly found in the small intestine, bile duct, and gall bladder of their bat hosts. Larval cestodes have been reported relatively infrequently from bats.

Protozoans of the genus *Eimeria* are generally found infecting the epithelial cells of the villi lining the small intestine, and the oocysts are passed in the feces of their host.

To insure complete necropsy and to locate parasites wherever they may occur in the specimen bat, every part of each prospective host to be investigated should be processed and examined in some way. Detailed information on collecting techniques can be found in Anderson (1965), Pritchard and Kruse (1982), and Gardner (1996).

METHODS FOR COLLECTING ENDOPARASITES FROM LIVE BATS

The following methods should be used to collect endoparasites from live bats captured in the field (for subsequent release) or maintained in captivity. It is important to note that at the present time, it is not possible to identify very many of the metazoan endoparasites of bats to the species level without euthanizing the bat host in which the parasites reside.

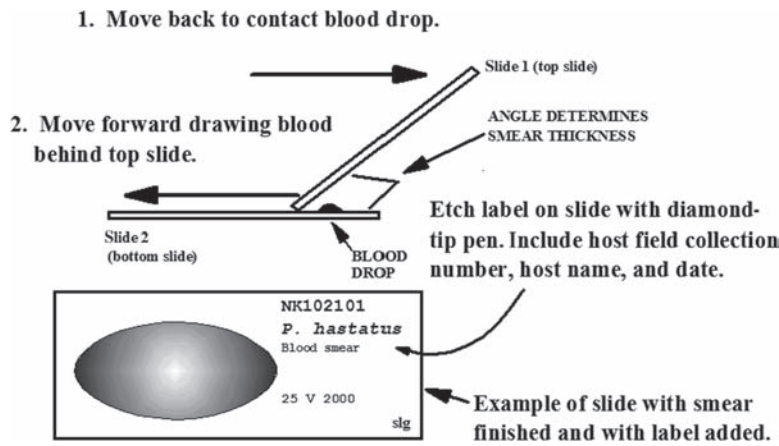


Figure 39.1. Method for preparation of blood smear. The smear must be made from fresh, uncoagulated blood. Note that the angle of the slide above determines the thickness of the smear. A steeper angle creates a thinner smear, while a shallow angle provides a thicker smear. Approximately 45 degrees is a good general angle for the top slide.

1. Bats should be subdued either with light anesthesia or by holding.
2. The uropatagium and wing membranes should be examined with a bright spot lamp held behind the membrane, and the membrane should be examined from the opposite side using a 2× to 5× Optivisor magnifier. Usually, any cysts containing nematodes will be 1–5 mm in diameter.
3. The area of interest is noted in the field catalog and is photographed with a digital camera to track cyst development over time. The area is disinfected by rubbing a cotton-tipped swab (Q-Tip) with Betadine solution. Any cysts located should be gently opened with a sterile dissecting pin and the nematode within transferred to a saline solution. The specimen(s) should be processed following the procedures outlined below.
4. Infection of a bat with filarioid nematodes, trypanosomes, or other blood protozoans is most easily determined by making a blood smear on a standard microscope slide from some peripheral blood. In this case, a blood smear should be made by taking a small amount of blood from the wing membrane or uropatagium and introducing this onto a microscope slide (see below and Fig. 39.1) and processing for later microscopy.
5. To determine if the bat is infected with gastrointestinal helminths or protozoa, a sample of feces should be saved in a vial filled one-half full with a 2% Potassium Dichromate (water v/v) solution. The bat will generally defecate while being handled; these feces should then be collected, so care must be taken to ensure that the sample is obtained without cross contamination between individual specimens of bats (change surgical gloves after handling each sample). Insert a tag (Fig. 39.2) into each vial with the field identification number.

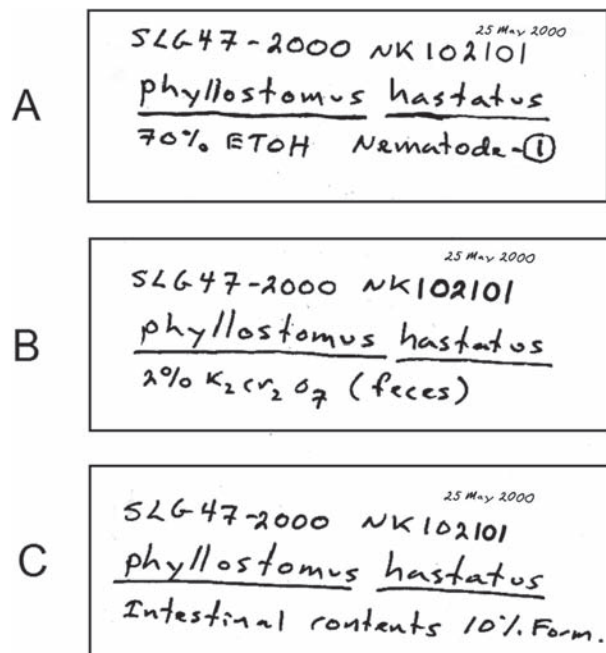


Figure 39.2. Examples of data tags to be placed in vials or plastic bags with specimens of parasites or fecal samples to be examined later for the presence of endoparasites (in this case, coccidian oocysts). A, tag for vial with a single nematode preserved in 70% ethanol recovered from the small intestine of *Phyllostomus hastatus* NK102092. B, tag for a vial containing feces from the lower bowel of *P. hastatus* NK102092, in potassium dichromate. C, tag for a vial containing intestinal contents (three cestodes) in 10% formalin from *P. hastatus* NK102092. The collector's number, the field identification number, the kind of preservative, and the general kinds and approximate number of parasites in the vial or bag are noted on each tag.

6. After the fecal sample is collected and stored in dichromate solution, the specimen should be kept at or less than approximately 21°C before being processed. It is beyond the scope of the present paper to discuss analytical techniques beyond normal collection and preservation; however, the goal of this part of

the collection protocol is to obtain eggs or protozoan oocysts that are being produced by the parasites living in the lumen or the lining of the gastrointestinal tract. For research purposes, these eggs or oocysts may be identified to the species that produced them using morphological methods and perhaps via advanced molecular techniques. However, as indicated earlier, the molecular, morphological, and phylogenetic database is not yet developed enough to enable this kind of analysis. Note that we encourage collection, preservation, and vouchering (in recognized museums) of these data for future analyses.

METHODS FOR COLLECTING ECTOPARASITES FROM MUSEUM SPECIMENS

The following procedures should be used for the collection of Protozoa (Coccidia), Acanthocephala (thorny headed worms), Cestoda (tapeworms), Trematoda (flukes), and Nemata (nematodes) from bats prepared as museum specimens.

1. *Preparation for Endoparasite Collection.* Researchers should collect endoparasites from the host after it has been euthanized and before other tissues have been removed to be used for future genetic analyses. Always rinse scissors, forceps, and probes in 70% ethanol made with a solution of 10% sodium hypochlorite (bleach) and dilute high-phosphate detergent, rinse in distilled or purified water, and wipe dry with a clean unused tissue between animals to avoid contamination with blood or other sources of foreign DNA. To make sure that no cross contamination occurs, this part of the protocol needs to be followed closely.

2. *Collection of Blood and Preparation of Blood Smears.* If the animal is still fresh (i.e., the blood has not coagulated) or alive, a blood smear is made. Blood can be collected from any convenient area from a dead animal, usually while taking tissues. Blood can be collected using a hematocrit tube (see Voigt et al., this volume). If it is collected with the tube, it can either be sealed with critoseal and stored (for future use in molecular analysis of blood protozoans) or transferred immediately onto a slide. Typically, the investigator collects the blood using a disposable 1-mL plastic or glass pipette and then places a drop of blood (approximately 250 μ L) in the middle of a slide. The edge of another slide held at an angle is placed on the slide with the blood so as to contact the blood drop. The inclined slide is pushed evenly and rapidly away from the blood drop, drawing the blood out into a thin (one cell layer) smear (Fig. 39.1). Label the slide using a diamond-point pencil with the field identification number of the bat and allow the slide to dry. Depending on humidity, the slide should dry for a period of 10–30 minutes. New blood smears are fixed at the end of each day (or sooner after drying if the temperature and

humidity are high). Blood smears are fixed in 100% methanol for 2–5 minutes. Blood smears that are well formed and of even thickness are important for documenting and detecting hemoprotozoa and microfilariae (juvenile nematodes) that occur in the bat being studied.

3. *Searching for and Collecting Endoparasites.* To search for and collect endoparasites from the digestive system, the investigator opens the abdominal cavity and cuts through the esophagus just above the stomach and through the colon, just anterior to the rectum. Care must be taken not to perforate the organs during this procedure because of the possibility that parasites may transfer (or appear to transfer) from one organ to another, thus invalidating information being collected on distribution of parasites in one individual bat. The digestive tract is removed intact and is placed in a clean Petri dish (plastic or glass) with a tag (Fig. 39.2) bearing the field identification number of the bat and a generous amount of water or preferably, physiological saline. If the organs are large (being derived from any of the Megachiroptera), porcelain pans are used in place of Petri dishes and beakers.

4. *Examining the Host for Endoparasites.* During this step, the researcher should examine the body cavity, liver, kidneys, and lungs for helminth cysts or filarioid worms. All organs to be removed from the bat for future molecular or biochemical-genetic work must be carefully examined for parasites. Filarioid nematodes may be encountered in the heart, aorta, pleural cavity, mesenteries, or subcutaneous tissues. Larval or juvenile acanthocephalans, pentastomes, nematodes, cestodes, and trematodes may be found in the liver, mesenteries, or any other tissues.

In the unlikely event that cestode cysts are encountered in the bat being studied, some are fixed in situ in the bat tissue. This is accomplished by removing part of the organ (muscle or liver) with the cyst intact and preserving the sample in 10% formalin. If more than about 10 cestode cysts are encountered in one host, some cysts are removed and carefully cut open, and the cestode strobila is relaxed in distilled water and then fixed in 10% formalin. In addition, some are stored in 95% ethanol, and some are frozen in liquid nitrogen. These various methods of preservation ensure that adequate material will be available for future investigations of both morphology and molecular markers such as the DNA sequence coding for cytochrome oxidase I or other molecules (ITS-1, etc.) for DNA bar coding (Marshall, 2005).

5. *Examination of Organs for Endoparasites.* After searching for parasites in the body cavity and other tissues of the bat, the collector frees the intestinal tract from attached mesentery and straightens it by gently cutting away at the mesenteries with blunt-nosed (iris or strabismus-type) scissors. This step is necessary to speed the process of cutting the length of the intestine with the blunt-nosed scissors. Stomach and intestines are placed in separate Petri dishes or containers, each with a tag bearing the field

identification number of the bat. Petri dishes must always be cleaned with detergent and water, rinsed with distilled water, and dried between uses (thus we advocate the use of disposable plastic Petri dishes). Each organ is opened separately and examined separately for parasites. Again, it is important to use scissors with blunt tips, because scissors with sharp points perforate the organ while cutting, making it difficult to open the organ quickly. It is crucial to have a good source of illumination both underneath and over the specimen to be necropsied. We recommend using portable fluorescent workshop lights; these pull a lower number of amps relative to incandescent bulbs, which is important when generators are in use in field situations. When no electrical power or generator is available, headlamps and other portable battery-powered light sources must be used. Again, it is essential that adequate bright light is available during necropsies. Headlamps with light-emitting diodes are now common and work well in place of incandescent and fluorescent lighting. They operate with very low current drain and enable the researchers to extend battery life, which is very important in remote field situations or small field expeditions. If an abundant supply of water is available, intestinal contents can be washed in a soil screen and then placed in a Petri dish and searched under magnification or with a dissecting microscope. The screened material should be examined in relatively small aliquots to make sure that there is not too much intestinal content debris obscuring any helminths that may be present. If water supply is limited, one can take advantage of the fact that worms sink and other materials float. The intestinal contents can be covered with water (if large) or physiological saline 9% (if small) in a Petri dish and gently stirred. The lighter materials (plant parts and other food items) that float or do not settle quickly can be decanted. If the bat is very small, this is not necessary and the intestine can be opened in a small Petri dish in saline and examined directly with a dissecting microscope. For large specimens, the decanting procedure is repeated several times, the intestine is removed from the Petri dish, and the remaining material is searched for helminths. Searching is conducted with either a dissecting microscope or a 10×jewelers magnifying visor (we like the Optivisor, but there are many others that work as well). Huge numbers of extremely small trematodes and nematodes can occur in the small intestine. If many helminth specimens are encountered while searching, the intestinal contents should be preserved in 10% formalin so accurate parasite counts can be made in the laboratory (and see step 6 below).

Organs such as skin, eyes, urinary bladder, lungs, gall bladder, and nasal cavities must also be examined, preferably with a dissecting microscope. Small trematodes commonly occur in the bile ducts and gall bladder of the liver and the mesenteric veins, and small nematodes can be found in the urinary bladder and in the lungs. Mites can

be found in the subdermal area as well as in the trachea, lungs, nasal passages, and in other tissues. Coccidia (genus *Eimeria*) have been recovered from the liver of bats using tissue sectioning or tissue smear techniques.

A tissue smear is made by cutting through the tissue in question (spleen, liver, etc.), holding a part of the tissue with a pair of forceps, and touching the tissue section several times down the length of a cleaned microscope slide, being careful not to touch the same spot on the slide more than one time. The free cells in the tissue adhere to the slide wherever it is pressed against the dry slide. The slide is then treated (fixed and preserved) following the procedure for blood smears (step 2).

6. Fixing and Preserving Endoparasites. To relax cestodes (tapeworms), trematodes (flukes), and acanthocephalans (thorny headed worms) one places them in distilled water, although tap water or filtered river, pond, lake, or stream water may be used. Water (but not saline) is also used for killing and relaxing specimens of platyhelminths or acanthocephala. Placing the worm in water creates an osmotic imbalance, which causes water to move into the body tissues and cells of the worm, leading to osmotic shock and ensuing death (Note: If one wishes to keep the specimens alive for a time before fixation, the use of physiological saline is recommended). The increasing pressure within the body tissues also may cause the rostellum on the scolex of a cestode or proboscis (acanthocephalan) to evert, which is necessary for identification of most (if not all) of these parasites. The strobila of a cestode relaxes when the animal is exposed to purified water, i.e., water that is hypotonic relative to the tissues of the worms. It is especially important to leave a specimen in water long enough for eversion of the scolex or proboscis and relaxation of the strobila or body to occur. (Note that we have processed hundreds of thousands of parasites in this way, including performing complete necropsies on more than 10,000 mammals and birds. We have not seen any loss of hooks from worms using these techniques. In our experience, the loss of rostellar hooks from the scolex of tapeworms from birds or mammals is always caused by death and autolysis of the parasite within the host. It is necessary to remove the intestines from the host within 1–2 minutes of time of death or poor results may be obtained.) The relaxation of the strobila (or body of the cestodes) is a process can take less than 5 minutes to more than 1 hour depending on the size of the worm, the ambient temperature, the salinity of the water used, and the species. After being relaxed and killed, helminths to be saved for morphological study are fixed in hot, (not boiling) 10% buffered formalin solution (see below) and placed in vials with both the field identification number and the location of parasite in the bat written on the tag, e.g., SI for small intestine, LI for large intestine, GB for gall bladder, etc.

We also recommend using 10% buffered formalin for fixation (assume for fixation purposes that 37% formalde-

hyde is equivalent to 100% formalin for the 10% dilution; see recipe for buffered formalin in Humason, 1979). Paraformaldehyde can be used in place of formalin, and some feel that better fixation is achieved with this chemical, especially when considering transmission electron microscopy (Humanson, 1979). When the specimens are ready to be fixed, place them in a vial with sufficient hot water (or saline), adding enough 100% formalin to make a 10% solution. Alternatively, the parasites can be placed in Petri dishes and hot or cold formalin can be poured on top of the specimens. Parasites from each organ must be preserved in separate vials, and parasites from one organ should not be placed with the parasites from another organ.

Platyhelminths (cestodes and digenes) can be relaxed and killed in a number of ways. The first method uses cold distilled water relaxation then immersion in hot 5% to 10% buffered formalin solution. We have excellent results using cold formalin as long as the cestodes and trematodes have been well relaxed in distilled water first. Additionally, hot formalin may be dangerous in a field situation, so plan your work accordingly.

Saline is never used to relax cestodes or other platyhelminths because it prevents osmotic imbalance in both and therefore keeps the specimens from relaxing before fixation. Nematodes, on the other hand, should be placed in saline at time of dissection, and distilled water should be avoided. Osmotic imbalance caused by immersing nematodes in distilled water usually causes the nematode to burst, and in many cases the specimens are destroyed. While working with the bat, nematodes can be placed temporarily in saline and then transferred directly to a vial filled to the 90% level with very hot water (not boiling). Make a 10% formalin solution in the vial by filling the vial the rest of the way with 100% formalin. Alternatively, the nematodes can be placed in glacial acetic acid (GAA) for a few minutes before being transferred to either 10% formalin or 70% ethanol. (*Note:* The GAA method for uncoiling nematodes does not work for the nematodes that live in the stomach, because they are already exposed to a low PH environment.) The GAA treatment causes most nematodes to uncoil and straighten, and they can then be stored in either 70% ethanol or 10% formalin. Specimens treated following these directions are much easier to identify than those fixed without straightening, because the morphological characters are more readily seen. Specimens to be saved for molecular analyses should be washed in saline, placed in a cryotube (1.5 mL), and stored in liquid nitrogen or placed in a vial in 95% ethanol, and any exposure to formalin or acids should be avoided.

7. *Collection of Samples for Genetic Analysis.* If sufficient numbers of helminths are available, representative individuals should be preserved in liquid nitrogen for future DNA studies. It must be stressed that because glacial acetic acid and formalin will severely damage or destroy the DNA of organisms, individuals preserved for future gene-

tic analysis must not be treated with GAA or formalin before being frozen or preserved in ethanol. Alternatively, specimens to be saved for studies of DNA can be preserved in 95% ethanol. Because of the vagaries of transport of specimens from remote field locations, the researcher should preserve parasites using many different methods, thus insuring availability of adequate material for future studies. DNA extractions should be performed relatively soon after samples are brought from the field; storing samples in 95% ethanol in the refrigerator or freezer may decrease the chances of obtaining DNA of good quality at a later time. If refrigerated at 2°C, DNA from specimens so stored may be viable for only 2 to 3 years; therefore, either long-term storage at -85°C or permanent cryo-storage in liquid nitrogen preservation is preferred.

8. *Collection and Examination of Feces for Endoparasites.* Fecal material collected from the bat should be preserved in a 2.0% potassium dichromate ($K_2Cr_2O_7$) solution for later concentration, collection, and study of coccidia and (possibly) helminth eggs. A fecal pellet or some material from the rectum should be placed in a vial filled approximately half-full with 2.0% $K_2Cr_2O_7$, with a tag bearing the field identification number and the genus name of the bat. Wheaton snap-cap vials (15 mg) are best for this because one vial filled fully with 2.0% $K_2Cr_2O_7$ contains a sufficient quantity of oxygen to keep the coccidia alive, they are reusable many times, and they very rarely leak.

9. *Morphology of Gastrointestinal Tracts.* If many conspecific bats are available, two to five entire gastrointestinal tracts should be preserved individually in 10% formalin. This will allow future examination of the morphological characteristics of the intestines and any worms associated in situ. The examination will also allow the researcher to perform the necropsy in a more relaxed way in the appropriate settings of a modern laboratory (in contrast to sometimes less than adequate field conditions). However, it should be noted that any recovered cestodes will probably be unidentifiable due to poor relaxation and distortion of the specimens.

10. *Freezing Tissues in Liquid Nitrogen.* Depending on the size of the bat, time constraints, and availability of liquid nitrogen storage containers, complete digestive tracts can be removed and frozen. We have had very good results with this method, and have recovered trichostrongyloid nematodes that were still osmotically active (indicating intact cellular membranes) after the intestines were frozen and stored for several months in liquid nitrogen. This method of collection will enable the researcher to retain almost all of the data available in a very convenient and efficient manner in a single step. Coccidian parasites and helminth eggs can be preserved in 2% potassium dichromate (see step 9, above) by taking a fecal sample from the posterior part of the large intestine before the rest is frozen. This will allow the researcher to have an idea of whether the sample that is in the freezer has a certain probability

of containing helminths in the GI tract. (If eggs are found when the fecal sample is examined, it is 100% certain that the intestine contains helminths, but lack of eggs in the sample does not preclude the possibility of finding helminths when the intestine is examined.)

11. *Fixing Parasites for Microscopic Analysis.* In some cases, the study of fine morphologic features requires the use of specialized microscopy such as confocal or electron microscopy. Scanning electron microscopy (SEM) is commonly used in parasite taxonomy because it allows resolution of minute external structures of the worms. A concise summary of the discoveries made using modern microscopy may be found in Halton (2004).

Because of the importance of discoveries made using microscopy, it is recommended that specimens be fixed when they are still alive, since washing is essential to eliminate bacteria and debris covering external surfaces of specimens under study. There are several methods available to preserve materials for electron and confocal microscopy, and depending on the group of helminths, some may be more appropriate than others. The researcher needs to find the best method relative to its feasibility under field conditions and amount of time required to complete the task. Most of the methods require time as well as proper and safe facilities (i.e., fixing specimens using glutaraldehyde requires a hood, ice, water, and glassware). For those reasons, we recommend using either paraformaldehyde or formalin, which preserve specimens without inducing much deformation. These fixatives are also relatively easy to manipulate in the field, and using them allows additional postfixation procedures to be performed if necessary.

If other fixatives are needed, it may be necessary to keep specimens alive long enough to transport them back to the laboratory. This can be done by storing one worm in an unused new vial with physiological solution and transporting the vial on ice (taking care not to freeze the specimen). Again, in most situations, this would not be possible and fixing the specimen and then performing a postfixation processing would almost always be the best alternative. Liquid nitrogen storage and transport using dry shippers is a very good alternative.

Using a solution of 10% formalin after washing the specimens in saline solution usually produces satisfactory results for SEM. Formalin may induce some shrinking of the whole body and of the organs. To avoid shrinkage, samples to be processed for special microscopy should be stored at 2°C before postfixation. Another drawback of using formalin is the hazard associated with transporting this volatile fluid. Paraformaldehyde has the advantage of being a solid, which is easier to transport, however the preparation may be more difficult, especially because it is necessary to dissolve the powder with other reagents. Paraformaldehyde preserves the antigenicity of the proteins better than formalin and glutaraldehyde, which may

Bolivia, 2000	⑥
S.L. Gardner Field Catalog	
Bolivia: Beni; Little Rock, 3km S. Casarabe. 14°52'53"S.; 64°27'06"W. 190M (GPS)	
-25 May 2000-	
SLG47-2000 NK102101	<u>Phyllostomus hastatus</u>
Feces. Blood smear.	
Stomach ⊕, saved contents in whirl pak.	
Small intestine - anterior part (1/4)	
→ 1 small nematode - pres 70% ETOH in 1 dram vial.	
→ 2 small nematodes - pres 95% ETOH in 1 dram vial	
→ Approx. 10 small nematodes preserved in nunc tube - Lig. Nitrogen.	
→ 5 cestodes - Pres. 1 in Lig. Nitrogen	
4 relaxed in distilled water and preserved 3 in 10% formalin (1 dr. vial) and ① in 95% ETOH (1 dr. vial).	
→ Ectos preserved in 70% ETOH → whirl pak.	
-25 May 2000-	
SLG48-2000 NK102102	<u>Thyroptera</u> sp.
Feces, blood smear	
Saved whole G.I. contents and gut intact in cryotube (10ml) - Nalgene in Lig. N ₂ for later study in lab.	
- No ectos.	

Figure 39.3. Sample field catalog entries for process of recovering parasites from a bat. The collecting locality is always included at the top of every page, or more frequently on the page if specimens are processed from different localities at approximately the same time. Date is included with each record. Field collector number is next, followed by the field identification number (for data tracking) and field identification of bat. Record next the collection of feces and blood or organ smear. For SLG47-2000 NK1102101 *Phyllostomus hastatus*, the present entry indicates that the stomach had no parasites but that contents were saved for later analysis of diet. The record indicates that one small nematode was found in the anterior part of the small intestine and was preserved in 70% ethanol in a 1-dram vial. Twelve additional nematodes were found: two were preserved in 95% ethanol and ten were preserved in a cryo-tube (Nunc tube) in liquid nitrogen. Five cestodes were found: one was preserved in liquid nitrogen in a cryotube; four were relaxed and killed in distilled water, three of these were then preserved in 10% formalin in a 1-dram vial, and one was preserved in 95% ethanol in a 1-dram vial. The record also shows that ectoparasites were encountered and were preserved in 70% ethanol in a whirl pak. For SLG48-2000 NK102102 *Thyroptera* sp., the entry indicates that feces were taken and preserved in potassium dichromate, a blood smear was made, and the whole gastrointestinal tract was preserved in a 10-mL cryotube (Nalgene) in liquid nitrogen. No ectoparasites were noted at the time of necropsy. Negative data are always recorded to enable estimation of prevalence, intensity of infection, and other ecologically useful parameters.

be crucial in some confocal microscopy applications. The delicacy of preserving specimens for confocal microscopy using any fluid is that the pH of the fluid should be equilibrated with the intracellular pH. Standard formalin fixation works in some cases for confocal microscopy because formalin fixed tissues naturally fluoresce with certain wavelengths of the confocal microscopes.

12. *Recording Data.* Each researcher should maintain a field notebook in which information is entered on the specimens collected and the methods of preparation and fixation that were used. See Figure 39.3 for an example of a page from a parasitology field notebook. The notebook should have pages made from 100% cotton rag paper. Only permanent India ink or other nonvolatile ink should be used for recording data. Pencil is not recommended, because it can be erased. For each record, the field collection number of the host should be recorded as well as the researcher's catalogue number. Including the collection number with the parasite sample is necessary to allow accurate cross referencing with the voucher or symbiotype specimens (see Frey et al., 1992). Specimens should be numbered sequentially beginning with "1" and continuing indefinitely (for example, SLG-1 through SLG-12,500) rather than beginning the sequence anew with each collecting trip. However, specimens can be quickly identified to the year if the date is included on the collectors numbering system; for example, SLG47-2000 means that this is the 47th specimen examined in the year 2000 by Scott L. Gardner. The initials of the collector always precede the number in the notebook and on any tag created with his or her initials to allow identification of the collector at a later date. The date of collection and the locality of the collection must be provided for each specimen. The species name of the bat should be entered, while acknowledging the provisional nature of field identifications. The location of a parasite in a bat must be noted, as should negative searches (Fig. 39.3). The collector should also record the general types of parasites encountered in each organ.

MATERIALS NEEDED FOR PARASITE COLLECTION

Equipment and Glassware

Among the supplies required for the collection of endoparasites are the following equipment and glassware.

- Dissecting microscope with 0.5 × to 30 × magnification.
- Bright light source. Headlamps (e.g., Petzl DUO) running on four AA batteries work well, as there are two levels of power: high for detailed work, and low for general operation. These now come with LEDs that increase battery time and candlepower output.
- Two pairs of jewelers forceps (100 mm).
- Two pairs of gross dissection forceps with blunt noses (120 mm and 140 mm).
- Copelin staining jar for fixing blood smears.

- Small (300 × 200 mm) and large (400 × 300 mm) porcelain dissection trays.
- Scissors, both sharp nosed and blunt nosed of different sizes: 100 mm iris scissors for fine work, 120 mm blunt-nosed scissors for coarse work, and 120 sharp-nosed for tissue cutting.
- Rapidograph pens with indelible "India" ink or disposable Black UNIBALL Deluxe (Faber-Castell) permanent ink pens.
- Scalpel and disposable scalpel blades for cutting through bat tissue (size 21 is best).
- Small soil-sampling sieve (20-cm diameter; USA standard sieve series No. 325 [45 μm]) for catching the nematodes and allowing the small colloidal particles in the water to escape.
- Diamond-point pen for scribing field collection number on blood smear slides.
- Fluorescent workshop light for detailed work on microscope or with visor. These lights have a separate transformer located on the cord remotely from the light itself, therefore the light does not become overly hot during use (advantageous in hot climates and less so in cold climates). For an equal lumen output, fluorescent lights use much less current than an incandescent bulb. Work lights consisting of arrays of light-emitting diodes (LEDs) have recently become available and may further reduce dependency on generator or battery power.
- Liquid nitrogen tank (capable of static holding for at least 2 weeks or length field trip).

Expendable Supplies

The disposable items listed below are also among the supplies necessary for collecting endoparasites.

- Microscope slides
- Small, medium, and large plastic Petri dishes
- Small insect pin probes
- Dissection probes and needles
- Many disposable plastic pipettes
- 100% rag field notebook paper
- Vials, 15-mL or 20-mL snap-cap Wheaton vials
- Vials, 1-dram screw cap vials with Teflon inserts in cap
- Vials, 15-mL screw cap vials with Teflon inserts in cap
- Cryotubes (use brown lid inserts to indicate parasite samples)
- Labeling paper for inside the vials (can use 100% rag notebook paper or museum quality label stock)

Reagents

The following reagents are used directly or in the preparation of other solutions.

- 100% methanol (MeOH)
- 95% ethanol (EtOH), which can be used to make 70% EtOH

—-1
—0
—+1

- 100% formalin (37% Formaldehyde)
- Buffering salts for preparation of formalin or paraformaldehyde
- Paraformaldehyde, dry powder
- 2.0% potassium dichromate ($K_2Cr_2O_7$)
- Bleach, (sodium hypochlorite)
- Lysol disinfectant
- High-phosphate concentrated detergent to eliminate DNA from tools

ESTIMATED USE OF MATERIALS AND SUPPLIES FOR A FIELD COLLECTING TRIP

For general collecting, plan to use about one large plastic bag, two whirl-pak bags, and two each of 1-dram and 15-mL snap-cap vials per bat on each field collection trip. Actual use will probably be less because not all bats are infected with parasites.

Below is a list of disposable supplies needed for collecting parasites from approximately 100 bats.

- 1 box [144], 15-mL Wheaton snap-cap vials
- 1 box [144], 20-mL Wheaton snap-cap vials
- 1 box [144], 1-dram vials with Teflon-lined screw caps
- 2 boxes of standard precleaned microscope slides (not frosted)
- 10 boxes of tissues or Kimwipes for cleaning equipment
- 100 cryotubes with colored caps (use brown tops for parasites)
- 2 rolls of paper towels
- 200 disposable plastic pipettes
- 500 mL (100%) formalin
- 500 mL 95% ethanol
- 1,000 mL 70% ethanol
- 500 mL 100% methanol
- 500 mL 2.0% potassium dichromate ($K_2Cr_2O_7$)
- 200 rectangular plastic bags (1.5–2 mL thick and approximately 200 mm × 350 mm in size)
- 400 whirl-pak plastic bags with twist-tie closures (use only bags with stainless wire ties)
- 200 pages of 100% cotton rag field notebook paper

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