CHAPTER TEN

Methods for Specimen-based Studies of Avian Symbionts*

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Abstract. The collection of avian voucher specimens has long played an important role in studying the basic biology, ecology, and evolution of birds. However, symbionts (such as parasites and pathogens) of avian hosts have been largely neglected by ornithologists and are largely underrepresented in most major museum collections. Museum-oriented research expeditions to collect bird specimens capture a diversity of metadata, but the proper collection of symbionts for optimal use in downstream research projects remains uncommon. In this chapter, we provide methods for the comprehensive sampling of a diverse suite of symbionts from avian hosts, including blood parasites (haematozoans), microbial symbionts (bacteria and viruses), ectoparasites (arthropods), and endoparasites (helminths), while attempting to illustrate the research avenues opened by collecting such samples. Our objective is to encourage a view of birds as ecosystems in and of themselves, and to empower field ornithologists, particularly those participating in the collection of voucher specimens, to sample the plethora of micro- and macroorganisms that live in and on avian hosts. By collecting these additional specimens, ornithologists will not only unlock new aspects of avian biology, but also will expand the scientific community's ability to address ecological and evolutionary questions, while aiding in the discovery of new biodiversity and maximizing the utility of the "extended" avian specimen.

Key Words: field workflow, microbiome, museum collections, parasite, pathogen, symbiont, voucher.

Collections of avian specimens have been used to address complex ecological and evolutionary questions, and these museum specimens have served as an invaluable resource for the scientific community for centuries. As we develop new tools and methods, the scientific potential of individual bird specimens continues to expand, demanding that we take a more comprehensive approach to collecting modern whole bird specimens—considering them as ecosystems in-and-of themselves. Birds are capable of hosting a plethora of symbionts, some visible to the naked eye and others microscopic; some are ectoparasitic and some internal (Figure 10.1). Relationships between these symbionts and their avian hosts range from mutualistic to parasitic to pathogenic, and all have the potential to influence avian behavior, ecology, and evolution (Combes

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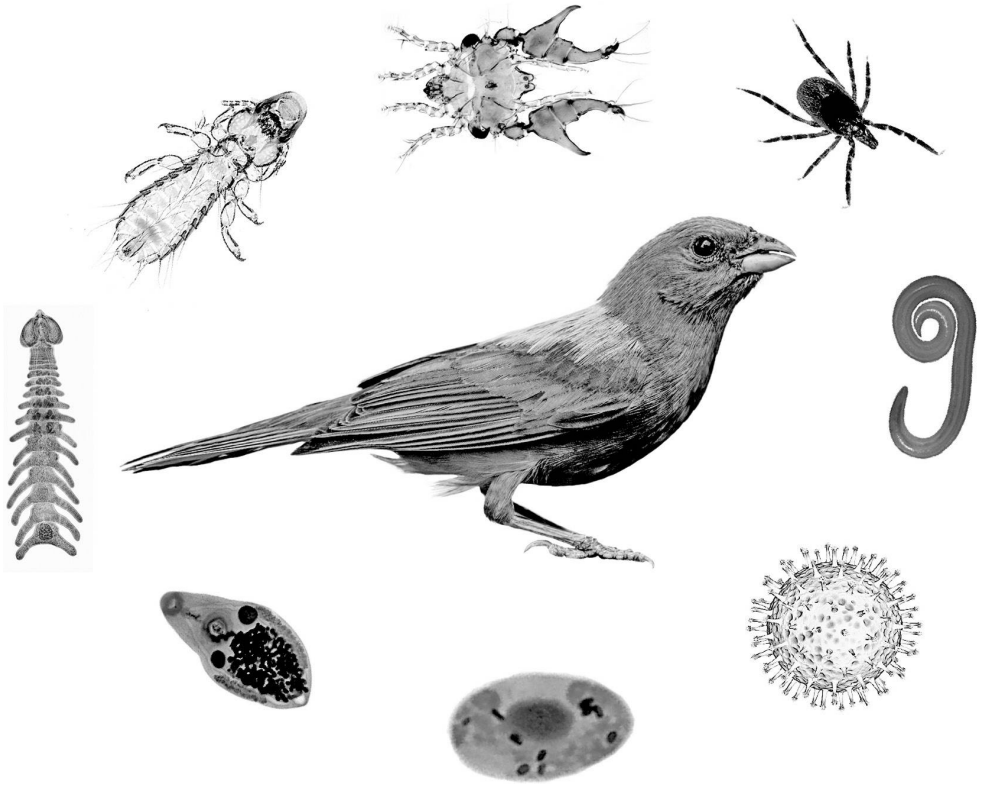

Figure 10.1. Birds can be thought of as ecosystems in-and-of themselves, serving as hosts for a plethora of symbionts from disparate branches in the tree of life.

1996, Combes et al. 1996, Atkinson et al. 2008). Indeed, studies of avian parasites and pathogens have allowed ornithologists to address many important questions, from understanding how avian life history traits are associated with higher or lower prevalence and probability of parasite infection (Clayton et al. 1992, Clayton and Walther 2001, Fecchio et al. 2011, Lutz et al. 2015) to tracking how avian populations have shifted their distributions over time. Because most birds are volant, they have also provided an important system for studying the evolution of virulence in rapidly spreading emerging pathogens (Hochachka and Dhondt 2000) and have brought to light the importance of broadly sampling potential hosts when studying the origin of epidemics (Kilpatrick et al. 2006, Dhondt et al. 2014).

Specimen-based studies of avian symbionts are particularly useful for studying cophylogenetic history and macroevolutionary patterns in avian hosts and parasites (Johnson and Clayton 2003a, Weckstein 2004, Johnson et al. 2011), as well as spatiotemporal relationships between birds and their environments (Parker et al. 2011, Galen and Witt 2014). For example, studies of museum specimens dating back to the early 20th century have allowed researchers to determine when avipoxvirus was first introduced into endemic Galápagos finches and mockingbirds (Parker et al. 2011) and Hawaiian forest birds (Jarvi et al. 2007). In separate studies, the sampling of haemosporidian parasites, which are the causative agents of malaria, across a broad range of avian hosts has led to generalizations about the power of host life history traits to predict rates of parasitism. For example, flocking behavior, nest type, and nest height or foraging stratum have been significantly linked to rates of parasitism for these sorts of malarial parasites in both the Neotropics (e.g., Fecchio et al. 2011, 2013) and Afrotropics (Lutz et al. 2015).

Haemosporidian parasites of birds have been studied for more than two centuries, and the knowledge we have acquired from these model

parasites has informed the study of human and other primate parasites that cause malaria. The incredible diversity comprising avian haemosporidians (Valkiūnas 2005), paired with the known selective pressure these parasites impose on their hosts (Samuel et al. 2015), make them an important group to consider when studying certain aspects of avian biology. In extreme cases, avian malaria can have devastating effects when introduced to naïve populations, as occurred on the Hawaiian Islands, where several species of Hawaiian honeycreepers were driven to extinction by introduced malarial parasites (Atkinson and LaPointe 2009). Extinction caused by malaria is an extreme and rare occurrence; in fact, by most outward measures of health, infected individuals appear to suffer little from malaria (Valkiūnas 2005). However, evidence suggests that subtle, long-term fitness effects are at play in wild birds: chronic malaria has been linked to telomere degradation and senescence in Great Reed Warblers (Asghar et al. 2015), as well as reduced quality of offspring and overall lower reproductive success of infected adults (Knowles et al. 2010). Such documented influences of microscopic blood parasites on avian hosts cannot be ignored when considering host ecology or evolutionary biology.

In some cases, parasites may reveal important information about the evolutionary history of their avian hosts. One of the nice attributes of ectoparasites, such as avian chewing lice (Phthiraptera), is that they are permanent ectoparasites, living their entire life cycle on the host (Johnson and Clayton 2003b). Another nice attribute of this system is that the life cycle of a louse from egg to reproduction is about 1 month (Johnson and Clayton 2003b) and thus a single annual cycle of the avian host contains 12 louse annual cycles. As a result, generation times of the parasites are much shorter than the host generation times, and thus the parasites evolve at a faster rate than their hosts (Whiteman and Parker 2005). Both of these characteristics allow ectoparasitic lice to serve as markers of recent host evolutionary history because the parasites are evolving more quickly than their hosts. Indeed, in some cases the DNA of ectoparasitic lice may serve as a better proxy of recent host evolutionary history than the host's own DNA. In the example that follows, we describe a specific instance where ectoparasitic lice infecting sympatric congeneric toucans in the genus *Ramphastos* can tell us a great deal about recent host evolutionary history.

Weckstein and colleagues have been collecting associated specimens of both *Ramphastos* toucans and their ectoparasites since the 1990s in an effort to understand both their cophylogenetic and cophylogeographic histories (e.g., Weckstein 2004, Price and Weckstein 2005). *Ramphastos* toucans in Amazonian Brazil include two overlapping species complexes, *Ramphastos tucanus* and *Ramphastos vitellinus*, each of which are geographically variable and form hybrid rings around the Amazon basin (Haffer 1974). At any given locality in the basin, both *R. tucanus* and *R. vitellinus* may host the ischnoceran louse species *Austrophilopterus cancellosus* (Weckstein 2004, Price and Weckstein 2005). Within the *R. vitellinus* species complex, geographic variation in coloration clearly shows a break across the mouth of the Amazon River in eastern Amazonia; this break is also indicated by the subspecific taxonomy of this complex, and it is clear that there is no ongoing gene flow between *R. vitellinus* subspecies across this riverine barrier. In contrast, *R. tucanus*, which shows east–west variation in coloration, does not exhibit a plumage coloration break across the mouth of the Amazon River (Haffer 1974). Instead, the eastern Amazonian subspecies *R. tucanus tucanus*, which has a reddish-orange bill, is found on both the north and south banks near the mouth of the Amazon River (Figure 10.2). Thus, one is left to wonder whether the absence of north–south variation in coloration in *R. t. tucanus* across the mouth of the Amazon River is due to ongoing gene flow or, alternatively, recent cessation of gene flow, such that there has not been sufficient time for divergence in plumage coloration to accrue. Genetic data from *R. tucanus* for mitochondrial DNA (mtDNA) indicates a break across the mouth of the Amazon, suggesting cessation of gene flow between north and south bank populations of *R. tucanus*. However, sequences of nuclear introns from north and south bank *R. t. tucanus* have similar or shared haplotypes, which is consistent with a history of either ongoing or recent cessation of gene flow (J. D. Weckstein, unpubl. data). In this case an additional marker might be useful for corroborating the mtDNA results and assessing the alternative hypotheses of ongoing gene flow or recent cessation of gene flow among these populations, because this cannot be tested with the nuclear intron data. Analysis of mtDNA

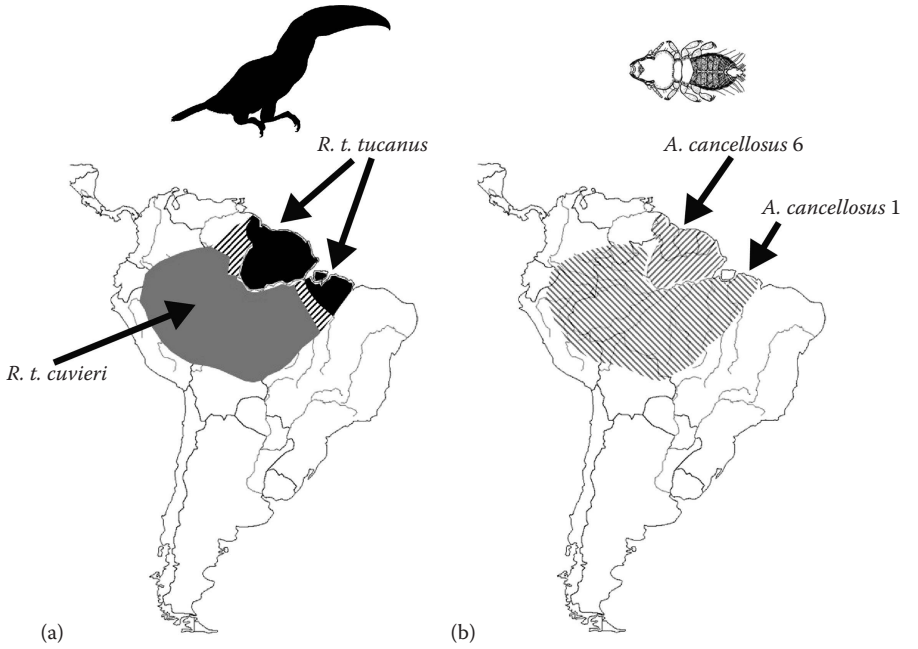

Figure 10.2. (a) Map showing the distribution of *Ramphastos tucanus* subspecies in Amazonia. Gray indicates the range of *R. t. cuvieri*, black indicates the range of *R. t. tucanus*, and hash marks indicate zones of hybridization between these subspecies. (b) Map showing the distributions of the divergent mtDNA lineages (Weckstein 2004) of the toucan louse *Austrophilopterus cancellosus*.

(cytochrome oxidase subunit I) sequences for the ischnoceran chewing louse *A. cancellosus* parasitizing these birds indicates a distinct genetic break across the mouth of the Amazon River, with louse individuals in the Guyanan shield differing by an uncorrected *p*-distance of 11.2% from those on the south bank of the river mouth (Figure 10.2). Thus, the lice are telling us that there is not ongoing dispersal of *R. t. tucanus* between the north bank and the south bank, corroborating the toucan mtDNA sequence data results and supporting the hypothesis that shared intron haplotypes between host populations on either bank of the Amazon River mouth are therefore the result of retention of ancestral DNA polymorphism on account of recent cessation of gene flow (J. D. Weckstein, unpubl. data). This example is simply one of many that highlight the value of making detailed collections of birds and their associated parasites. One could perform similar studies using myriad parasites with different life history characteristics to reconstruct the evolutionary history and ecology of their avian hosts.

Among avian symbionts, those with parasitic life histories, the major focus of this chapter, are particularly diverse (Windsor 1995), comprising an incredible 30% to 70% of known biodiversity on our planet (Timm and Clauson 1987, de Meeûs et al. 1998, Windsor 1998, de Meeûs and Renaud 2002, Poulin 2005). For a variety of reasons, parasites are important elements in the study of biodiversity (Combes 1996, Combes et al. 1996, Brooks and Hoberg 2000, Brooks et al. 2001, Whiteman and Parker 2005, Parker et al. 2006, Dobson et al. 2008). First, parasites can have important impacts on the health, demography, behavior, and evolution of their avian hosts (Combes 1996, Combes et al. 1996, Parker et al. 2006). Second, parasites are ubiquitous (Combes et al. 1996) with most, if not all, birds carrying many parasite species. For example, an individual bird can harbor lice, mites, ticks, hippoboscid flies, fleas, spiny-headed worms, tapeworms, flukes, roundworms, and protozoans, in addition to a plethora of bacterial, fungal, and viral symbionts. Third, only a fraction of the parasite species on Earth have been identified (Brooks and Hoberg 2001), and historically the effects of parasites on nongame wild avian hosts have been understudied (Parker et al. 2006, Atkinson et al.

2008, Atkinson and LaPointe 2009). Last, many parasites can be successfully used to make inferences about host ecology, population biology, and evolutionary history including historical biogeography (Whiteman and Parker 2005, Nieberding and Morand 2006, Nieberding and Olivieri 2007). Thus, a critical need exists to study the birds and their symbiotic associates (parasitic or otherwise) to understand the interdependencies in the web of life, reconstruct the evolutionary history of life on our planet, and stem the tide of extinction.

Biodiversity inventories of birds and their associated symbionts are a first step toward this end, and proper methods of collection and preservation are essential for correct identification and documentation of both host and symbiont species. A great deal has been written about the importance of avian biodiversity surveys (Lawton et al. 1998, Balmford and Gaston 1999, Norris and Pain 2002, Gregory et al. 2003) and methods for obtaining, preserving, and preparing bird specimens (e.g., Johnson et al. 1984, Proctor and Lynch 1993, Winker 2000). The importance of collecting avian specimens and voucher specimens in general has also long been acknowledged (e.g., Winker 1997, Rocha 2014). However, relatively little has been written regarding how to sample these host specimens for the high diversity of symbionts living on and in them. Although a number of publications have addressed collecting specific groups of parasites and other symbionts (Dubinina 1971, Clayton and Walther 1997, Owen 2011), these publications are scattered across the scientific literature. By far the most comprehensive description of procedures aimed at a complete parasitological investigation of birds was published by Dubinina (1971). This 129-page manual includes an overview of avian anatomy and morphology, step-by-step procedures for examining the entire avian host body for parasites, and directions for proper field fixation and postfixation protocols. However, this manual was published only in Russian and is now difficult to obtain. Furthermore, the introduction of modern research methods and tools since the 1970s has dramatically changed requirements for specimen fixation and preservation, leaving many of the methods presented in this work outdated.

Therefore, we outline here the general workflow, methods, and standards for comprehensive sampling and proper preservation of avian symbionts that we consider to be optimal for a variety of modern and traditional downstream biological research applications. Our goal is to optimize knowledge about each avian host and its symbionts, collected and prepared as traditional museum specimens, by broadly sampling four major symbiont categories: blood parasites (Haematozoa), microbial symbionts (bacteria and viruses), ectoparasites (arthropods), and endoparasites (helminths). The structure of this chapter reflects the order in which samples from these major groups are generally collected in the field workflow. We will not discuss the details of avian specimen preparation methods, as we assume that readers are already familiar with standard avian museum specimen preparation and data collection. If not, then the reader can refer to Winker (2000) or other papers cited earlier, which provide an overview of methods for preparation of bird specimens and the typical data fields that are recorded for each avian specimen. Following the final section on detailed protocols for symbiont sampling and preservation, we summarize a basic sampling workflow that can be applied in most field situations. We hope that this chapter provides a useful resource for avian collectors and field researchers, helping us edge closer to a more complete sampling of each avian "ecosystem."

BEFORE YOU BEGIN: THE FIELD NOTEBOOK

As with host specimens, careful field notes help to capture valuable metadata during avian parasite collection events. We use archival 100% cotton fiber, acid free paper with preprinted data fields and use archival ink (e.g., Pigma) to write notes on these "parasite field notebook" pages, which complement the host catalog notebook pages (Figure 10.3a,b). In these field notes, we record basic data such as host species sampled, locality, date sampled, parasite collector doing the sampling, and whether anything was found; it is also important to note when no parasites are found, so as not to be mistaken for a lack of sampling effort. The notebook pages use a series of checkboxes to denote what sampling was completed and leave room to describe what was collected from a given host specimen. One of the most critical data fields in this parasite field notebook is the host's field number, which is used to link parasite samples (e.g., vials of ectoparasites, blood smears) to a host voucher specimen. However, for this unique identifier to be useful for parasite sampling, it must be assigned to the host specimen *before* the

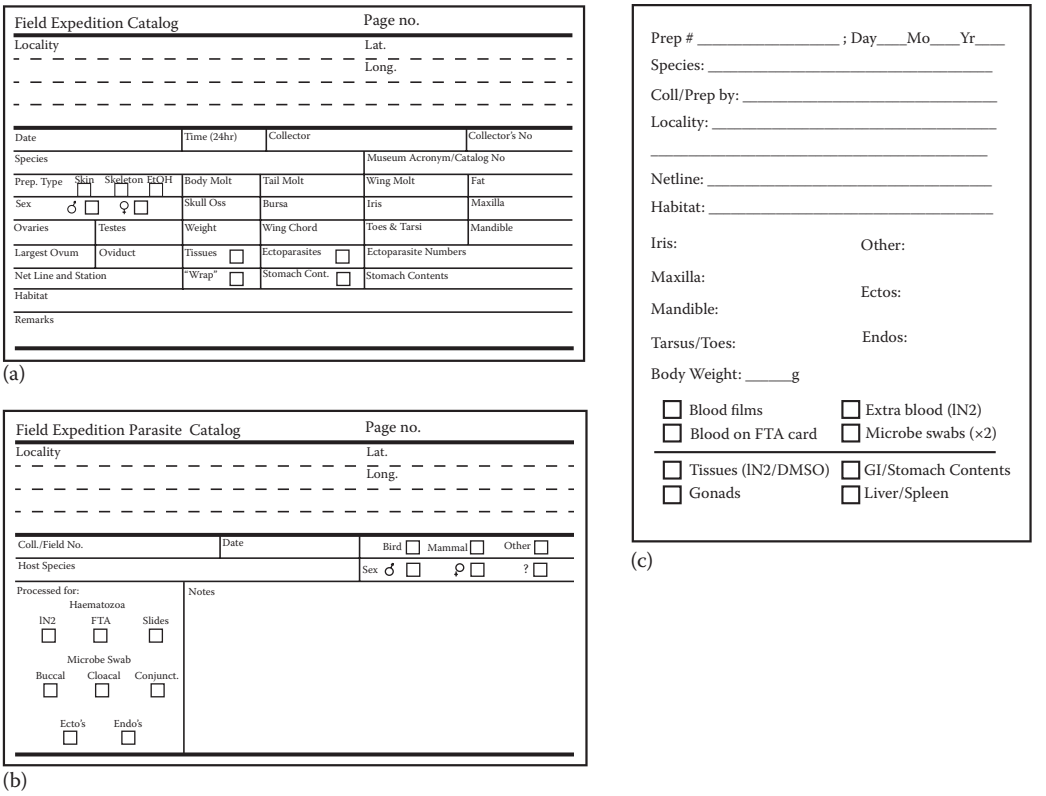

Figure 10.3. The field notebook. (a) Standard host catalog fields. (b) Example of parasite catalog fields. (c) Field sheet to be kept with host specimen as it goes through various stages of sampling and processing.

first parasite sampling begins, and thus before a field preparation number is usually assigned; often a personal catalog number is assigned by the host specimen preparator upon entry into the personal field catalog. In our experience there are multiple ways that this can be handled. One is to immediately assign a tissue or parasite number to each host specimen. Many museums use a separate tissue catalog to track the condition and handling of tissue samples collected in the field, and this number is noted on the voucher data label and host field catalogs. Another option is to assign either a special parasite field number or general host catalog number that follows the host specimen through all steps of sampling, and also note this number on the host field label, host field notebooks, and on a 10 × 15 cm host field sheet that follows the host specimen through parasite sampling and preparation (Figure 10.3c). This host field sheet is a convenient way to maintain notes on which sampling steps have been performed, as well as noting host data (e.g., weight, soft-part colors) before it is written in the catalog. We typically modify these sheets prior to expeditions to include a country acronym for collection numbers (e.g., "UGA" for Uganda), the year, and fields for specific tissues or samples we may be collecting for various projects.

SAMPLING PROTOCOLS FOR THE STUDY OF BLOOD PARASITES (HAEMATOZOA)

As with other taxonomic groups, the systematic study of avian haematozoans depends on both morphological and molecular data, both of which have their advantages and disadvantages. Phenotypic traits of haematozoan parasites may be convergent (Martinsen et al. 2008) and can be highly plastic depending on the host and the conditions during processing of blood smears (Valkiūnas 2005). Furthermore, haematozoan parasitemia is generally quite low in birds, which can lead to improper diagnosis of infection by microscopic analysis (Richard et al. 2002). The

development of molecular protocols has provided more reliable diagnostic methods and has led to the discovery of hundreds of novel haematozoan parasite lineages (Bensch et al. 2009). In addition to improving detection capabilities, molecular methods and the development of phylogenetic markers are proving increasingly useful for studying evolutionary relationships in the haematozoan tree of life (Perkins and Schall 2002, Martinsen et al. 2008, Perkins 2014). However, molecular data are prone to error in cases of multispecies infections, and, alone, are insufficient for the taxonomic description of novel parasites. Therefore, when sampling birds for haematozoan parasites, it is important to collect blood for both morphological and molecular analyses.

Blood Collection and Storage

For live birds, blood can be drawn immediately after recording soft-part colors such as maxilla, mandible, nares, eye-ring, tibiotarsus, and feet. This can be done alone or with the help of a partner, taking personnel experience and the size and vigor of the bird into consideration. The top priorities at this point should be proper handling of the live animal to reduce stress and suffering, and rapid processing of the blood sample once it has been drawn.

Blood from live birds can be obtained from several parts of the body, including the femoral artery, the brachial/ulnar vein, a clipped toenail, or in the case of shot or otherwise dead birds, directly from the wounds, body cavity, or heart. Although blood from a dead bird will still provide useful material for molecular analysis of some parasites, fresh blood is desirable for haemosporidian studies, due to morphological changes elicited in these parasites by a drop in temperature and/or exposure to air (Valkiūnas 2005). We have found brachial and jugular venipuncture to be the most efficient in both small and large birds, as these veins are easily visible, and, in most cases, can be sampled by one person working alone. Sampling blood by clipping the toenail should be avoided, as it frequently leads to the introduction of debris into the sample if not properly cleaned, produces a relatively low volume of blood, and may be quite painful for the animal. The toenail clipping method is not approved for most species by the Ornithological Council, Washington, DC (Fair et al. 2010).

For the majority of bird species, a small gauge needle (22–27 gauge) is best for sampling blood. Smaller gauge needles (larger numbers) reduce the likelihood of hematoma, but may increase the probability of hemolysis, affecting downstream hematocrit measurements and blood smear quality. We typically use 25 to 27 gauge needles. Be sure that your needles are designed specifically for subcutaneous use (frequently denoted "SubQ"), as other needle types (e.g., intradermal use) are blunt-tipped and inappropriate for venipuncture. Before searching for the vein, it is helpful to wet the area with alcohol or water to clear the feathers out of the way, which makes the vein more visible. Some researchers prefer to use petroleum jelly, which holds the feathers out of the way and causes the blood to bead up more effectively, making it easier to draw neatly into a capillary tube. We avoid the use of petroleum jelly due to its matting effect on the feathers of birds that are to be preserved as museum vouchers. The needle should be placed parallel to the vein, bevel side up. With very light pressure, insert the needle ~0.5 to 1 mm into the vein and quickly remove. A small drop of blood will then form and can be collected directly into a heparinized capillary tube. Do not place the capillary tube directly against the vein, as this can inhibit blood flow. Likewise, hyperextension of the wing or leg from which blood is being drawn can restrict blood flow. A typical microhematocrit capillary tube holds about 0.075 ml (75 µl). The volume of blood collected will depend on the size and condition of the bird, but for birds that are to be collected as specimens, 1 to 2 hematocrit tubes (0.075–0.15 ml) is more than sufficient for molecular and morphological analyses of haematozoan parasites (we often rely on <0.05 ml of blood for our studies). If the bird is to be released rather than collected, be sure to take no more than the equivalent of 1% of the bird's body mass in volume of blood (Fair et al. 2010) and check to make sure that the bird is in good condition and that it is alert before releasing.

Once drawn, blood should be stored for both microscopic and molecular analyses. Blood smears for microscopic analysis of parasite morphology should be prepared immediately after drawing blood (see next section). At this point, it is helpful to have a partner to whom you can hand the bird for euthanization. Alternatively, one person can bleed the bird and a second person can make

the blood smears. The person taking the blood sample can immediately euthanize the avian specimen after blood has been collected. See the Ornithological Council's guidelines for information on appropriate methods for euthanizing birds for preparation as museum specimens (Fair et al. 2010). Following the preparation of blood smears, multiple methods may be used for preserving whole blood for DNA studies: flash freezing blood in liquid nitrogen (the "gold standard"), storage of blood on Whatman® FTA® Classic Cards, and storage of blood in a DNA preserving buffer (e.g., Queen's lysis buffer, 95% ethanol). We typically place a small amount of blood on an FTA card for quick access in the lab, then store the remainder in liquid nitrogen for long-term storage in a cryogenic facility. Many researchers prefer to use 95% ethanol for the storage of blood for molecular analysis, as it is inexpensive and easily accessible in remote locations. Blood samples on FTA cards (or other filter paper), should be stored in a dry space free of contamination, such as a zip-closing bag with silica beads. FTA cards come with preprinted subsections for applying samples. Because we only require a small amount of blood for molecular analyses, we typically subdivide the cards using a custom-made stamp so that more samples may be stored on an individual card (e.g., we store nine unique blood samples instead of four).

It is important to note that unnecessary handling of birds can lead to a loss of ectoparasites, such as hippoboscid flies, which are volant and may leave the host when they sense a disturbance. Thus, it is best to quickly euthanize the avian host specimen, swab it for microbial symbionts (see following section), and then isolate the carcass in a plastic storage bag containing a fumigant for disabling associated ectoparasites. With this system, blood samples are rapidly prepared and the avian host is quickly relieved of suffering. It is very important to label both the host and blood samples (slides, vials, FTA cards, etc.) with a unique identifier (e.g., host field number or tissue number) before proceeding. This is particularly true if a large number of birds are in queue to be processed, or when multiple researchers are processing the avian host for different parasites and pathogens. Regardless of the circumstances, it is generally good practice to label specimen tubes immediately after sampling, and to tie a leg tag with this unique identifier directly onto the avian host immediately after it is euthanized.

Preparation and Fixation of Blood Smears

The quick preparation of blood smears is important for two reasons. First, the temperature change of blood can have profound effects on haemosporidian parasite morphology, making subsequent analyses of blood parasites complicated or even impossible. This may be linked to the life history of the parasite, with the temperature change simulating transfer of the parasite from the vertebrate to the invertebrate host, and inducing the progression of the parasite into the next stage of its life cycle (Valkiūnas 2005). Second, even when collected in heparinized microhematocrit tubes, blood can begin to clot, particularly in hot environments. If you are working alone and experience some delay before processing blood, it is helpful to first dab the end of the microhematocrit tube onto the FTA card (or other sterile paper, if planning to store blood in a lysis buffer) before applying a blood drop to the glass slides for preparation of blood smears. This removes any blood that has clotted at the end of the microhematocrit tube, allowing blood to flow more freely from the tube. As has been described in many useful guides (e.g., Gilles 1993, Valkiūnas 2005, Owen 2011), smears should be prepared on clean glass slides. Dust particles, grease, and scratches will significantly decrease the quality of your blood smear, and contaminated slides should be avoided. Unused slides that have been contaminated by dust or debris can be cleaned using ethanol and disposable wipes (e.g., Kimwipes manufactured by Kimberly-Clark) if necessary. A small drop of blood no larger than 3 mm in diameter is all that is needed to produce a good blood smear. A common error in the preparation of blood smears is the use of too much blood. The drop should be placed at one end of the slide, and a second clean "smearing" slide backed up at a 30 to 45 degree angle until it is touching the drop (Figure 10.4a,b), at which point the blood will spread across the back end of the smearing slide via capillary action. The smearing slide should then be pushed forward, briskly and smoothly, with blood trailing behind it (Figure 10.4c). If done properly, the blood smear should be in the shape of a bullet, with densest concentration of blood near the origin of the drop, and the edges of the smear feathering out toward the end (Figure 10.4d).

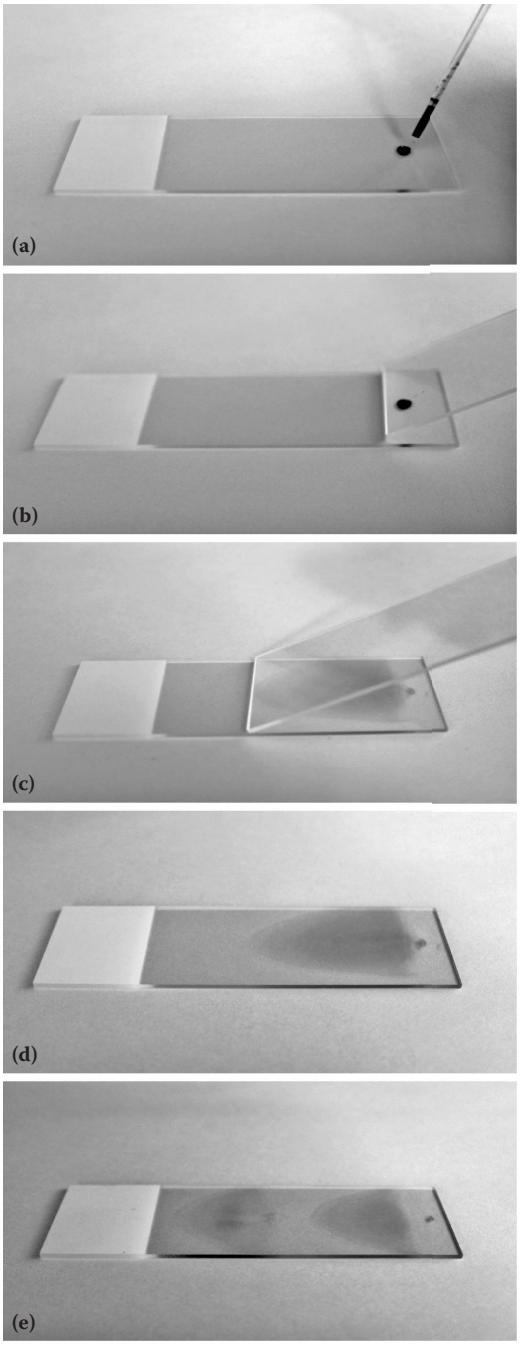

Figure 10.4. How to make a blood smear. (a) Place small drop of blood from microhematocrit tube near end of slide. (b) Back second slide up to drop of blood at a 30- to 45-degree angle. (c) Move the "smearing" slide quickly and smoothly across to spread the blood in a thin film. (d) Single thin blood smear. (e) Example of two blood smears prepared on a single slide.

It is important to produce multiple slides per individual when possible. Not only does this increase the number of fields that can be searched for haemosporidian and other blood parasites, but more important, it will allow for the deposition of slides at different institutions, which is often important when operating with collaborators. In the interest of maximizing the number of searchable fields, while maintaining the ability to share slides with different institutions, it is quite practical to produce multiple blood smears from the same individual on individual glass slides (Figure 10.4e). The ability to do this will vary with level of skill, environmental conditions, and the condition of the bird.

It is best to fix blood smears as soon as possible once they are air-dried, placing them in 100% methanol for 1 minute. If methanol is unavailable, it can be substituted with 96% ethanol and an extended fixation time of 3 minutes. Slides can be placed back-to-back in a Coplin jar containing fresh methanol. Replace the methanol frequently (every two to three batches of slides) to limit the effects of dilution and debris. Allow slides to air-dry face up, and once dry, place the slides back-to-back in a plastic slide box for storage. Alternatively, slides can be individually wrapped with paper, such as a Kimwipe, and bound together using rubber bands. Store fixed blood smears with silica beads, and stain as soon as possible. Although most staining agents containing methyl blue will allow for microscopic detection of Haematozoa, Giemsa remains the gold standard, and is the most commonly used stain for parasitological studies of haematozoan blood parasites. Rapid staining methods used for diagnosis of human malaria (e.g., Field's or Romanowsky stains) are less stable than Giemsa and prone to fading, and therefore are not appropriate for long-term storage and taxonomic studies. It is best to purchase a high-quality Giemsa stain and produce your own staining buffers (see Box 10.1 for formulas and staining protocol).

In many field situations, it is not possible to stain slides on the same day, or even within 1 week, of preparation (which is recommended). Older blood smears, if made and fixed properly in the field, are still useful for taxonomic research. However, it is a good idea to "refix" the slides once back in the laboratory by dipping them again in 100% methanol for 1 minute, and allowing to air-dry (R. Barraclough, pers. comm.). Older slides tend

Box 10.1 Giemsa Staining Protocol for Haematozoan Parasites

1. Prepare alkaline (N_2HPO_4) and acid (KH_2PO_4) stock phosphate buffers as follows:
   a. Buffer A: (9.50 g N_2HPO_4) + (990.50 mL dH_2O) = 1000 mL alkaline stock
   b. Buffer B: (9.07g KH_2PO_4) + (990.93 mL dH_2O) = 1000 mL acid stock
   c. Working buffer: (61 mL Buffer A) + (39 mL Buffer B) = 100 mL working buffer. The working buffer pH should be in the range 7.0 to 7.2.

   Stock buffers can be kept and reused to prepare a working buffer, which should be made fresh every few days. Stock buffers can be stored at room temperature indefinitely. Fresh working buffer should be made every few days and can be stored at room temperature as well.
2. Place a thin layer of high-quality Giemsa stain at the bottom of a Coplin jar, then add working buffer to produce a ~10% buffered Giemsa stain.
3. Slides should have already been fixed in methanol after blood smear preparation (in the field). However, it is a good idea to dip slides in methanol again before staining, particularly if they have been exposed to humidity, dust, and so forth. This additional methanol rinse will produce cleaner and more evenly stained slides.
4. Add slides to Coplin jar and allow to stain for 60 to 90 minutes. The duration of staining time will vary depending on the age of the slides, the quality of the Giemsa stain, and the concentration of the buffered Giemsa stain. Older slides tend to take up stain more readily and are likely to stain too darkly if left for too long. It is a good idea to test one or two slides before processing an entire batch.
5. Once staining is complete, remove slides from the Coplin jar and rinse off residual stain under water.
6. Slides should be labeled archivally, and the label should at minimum include the host voucher number. Once dry, slides should be placed in a secure slide box for long-term storage.

to absorb more stain, so Giemsa staining concentration and staining time should be reduced. It is a good idea to test your staining protocol on one slide before processing an entire batch. If the blood smear is overly dark and blue, simply dilute your stain or reduce the amount of time (add more stain or time if the slide appears too light). Once staining is complete, slides should be placed in a durable slide box and kept in a cool, dry environment for long-term storage. These slides will serve as vouchers and can be referred to at any point for morphological analyses of myriad haematozoan parasites found in avian hosts (Figure 10.5).

SAMPLING PROTOCOLS FOR THE STUDY OF MICROBIAL SYMBIONTS (BACTERIA, FUNGI, AND VIRUSES)

Studies of microbial symbionts in wildlife are in their relative infancy, and methods for sampling bacterial, fungal, and viral symbionts of birds are still being developed and improved. As our understanding of the interplay between microbial symbionts and avian evolution and ecology grows, so too should collections of samples from vouchered birds that are appropriate for studying these microbes (e.g., gastrointestinal tracts, fecal, buccal, and conjuctival swabs, etc.). The collection of such samples will ultimately provide important time series for the study of changes in microbial diversity in birds, which may allow researchers to measure the effects of environmental phenomena such as climate change and anthropogenic habitat disturbance, as well as the impacts of naturally occurring phenomena, such as dispersal and colonization, epidemics, and naturally fluctuating food cycles. Avian gut microbiota have probably received the greatest amount of attention (for a review of the current trends in this area of study, see Waite and Taylor 2015), and recent studies (e.g., van Dongen et al. 2013, Hird et al. 2015) provide useful methodological descriptions for studying the avian gastrointestinal microbiome.

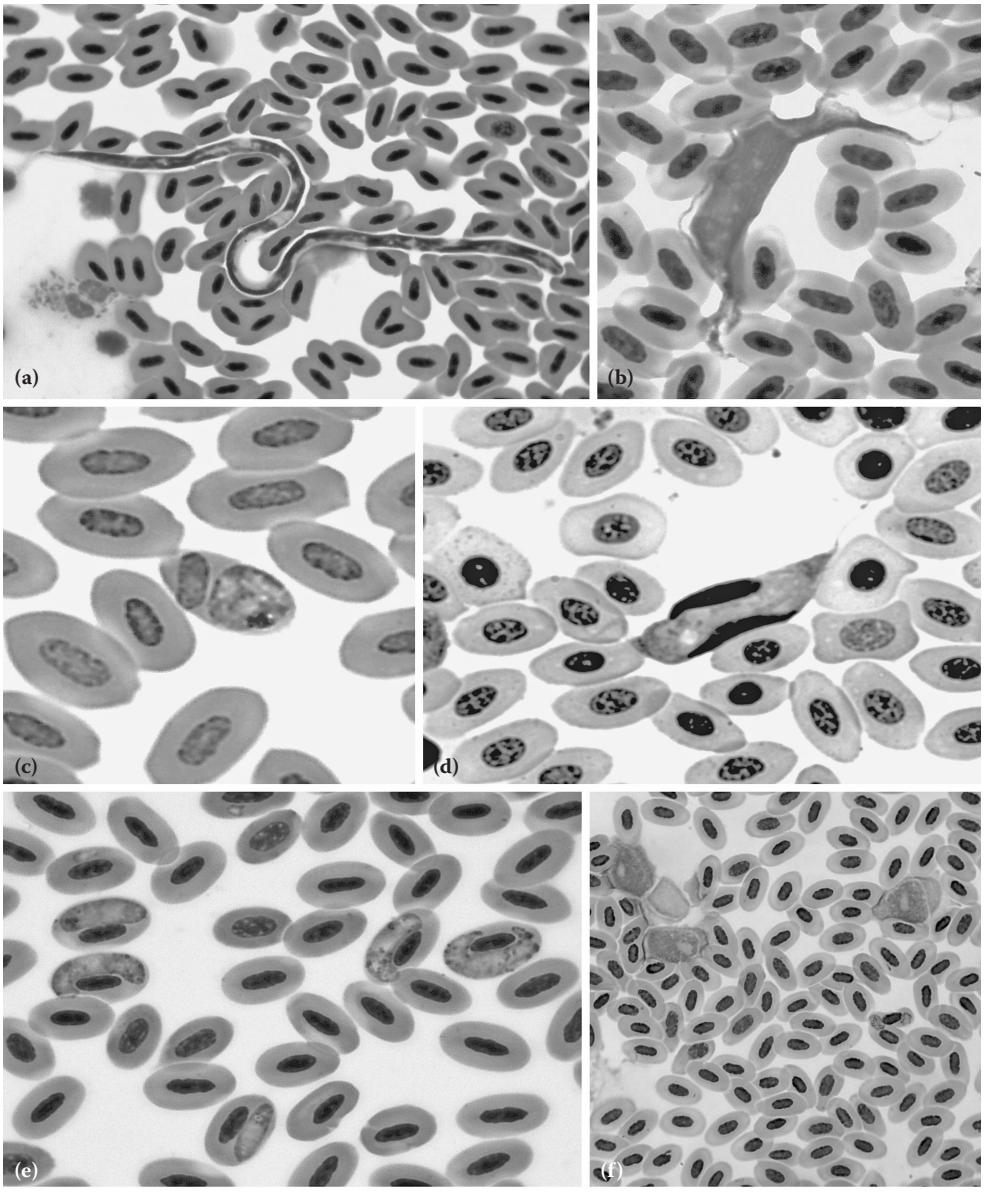

Figure 10.5. Microphotographs from Giemsa-stained blood smears of haematozoan parasites found in birds. (a) Microfilarial nematode ex *Pycnonotus barbatus*, Vwaza Marsh Wildlife Reserve, Malawi. (b) *Trypanosoma* sp. ex *Pycnonotus barbatus*, Vwaza Marsh Wildlife Reserve, Malawi. (c) *Plasmodium* sp. ex North American passerine. (d) *Leucocytozoon toddi* ex *Meleagris gallopavo*, Ithaca, NY. (e) *Haemoproteus* sp. ex *Ispidina picta*, Vwaza Marsh Wildlife Reserve, Malawi. (f) Coinfection with *Leucocytozoon* sp. and *Haemproteus zosteropis* ex *Zosterops senegalensis*, Nyika National Park, Malawi.

However, microbial symbionts are by no means restricted to the gastrointestinal tract, and other areas to consider when sampling a bird for microbial symbionts include the respiratory tract, conjuctiva, nares, and feathers.

As microbiome studies are still in their relative infancy, and best methods and practices are still being developed, we encourage researchers to check the most recent literature for proper sampling and storage techniques. Methods of preservation for the study of viruses in particular vary substantially, and knowledge of the viral family of interest is important when determining sample preservation methods. Because this area of study

is in a state of rapid development, we will provide here only the most basic advice on when and how to incorporate sampling for microbial symbionts into the field workflow, based on our experience.

Sampling for microbial symbionts (bacteria, fungi, viruses) should be conducted immediately after euthanization. Following euthanization, insert separate cotton-tipped sterile applicators into the (a) cloaca, (b) buccal cavity, and (c) conjuctiva, and rotate several times within each region to swab the area as thoroughly as possible. The applicator can then be placed into a sterile collection tube, the handle broken off, and the tube sealed. Proper storage of swabs will depend on the questions being addressed and resources available. Some options include immediate storage in liquid nitrogen, RNA*later*™, or other buffers (see Vo and Jedlicka 2014 for examples on downstream processing methods). We strongly encourage researchers to consider incorporating these simple and relatively inexpensive methods into their sampling regime, as few collections of microbial symbionts from wild birds currently exist, and the benefits and impacts of longitudinal studies are as yet undetermined.

SAMPLING PROTOCOLS FOR THE STUDY OF ECTOPARASITES

Birds are parasitized by a wide variety of ectoparasitic arthropods including, but not limited to, fleas, hippoboscid flies, lice, mites, and ticks (Figure 10.6; see Clayton and Walther 1997). Here we will focus on methods for sampling these parasites from dead avian host specimens collected during biotic survey field expeditions, although there are a variety of additional methods that can be used to collect ectoparasites from live birds and that can be used in laboratory settings (Clayton and Walther 1997, Walther and Clayton 1997, Clayton and Drown 2001). Clayton and Walther (1997) include a broad review of methods for quantification and collection of avian ectoparasites.

Fumigation and Collection of Ectoparasites

The first rule for collecting ectoparasites from avian host specimens is to not allow dead host specimens to come into physical contact with one another. Each freshly killed or caught bird should be isolated in a separate clean bag. Birds that are mist-netted can be carried back to the specimen preparation area alive using clean cloth bags. Bags should be washed thoroughly between uses to avoid potential contamination of parasites between individual birds. Birds that are shot can be placed immediately into a plastic storage bag with a note indicating soft-part colors (which may fade very quickly) and a cotton ball with a few drops of ethyl acetate on it. This will begin fumigation, allowing the bird to be immediately ruffled for ectoparasites upon arrival in the field camp. Upon arrival into camp each dead bird specimen in a bag should have a field number assigned to its field sheet (e.g., Figure 10.3c) so that the remaining host and parasite data collected from the bird can be linked to the voucher host specimen. Birds that are caught live will be euthanized after blood samples are collected. These birds should also be placed in a clean plastic storage bag with a cotton ball soaked with a few drops of ethyl acetate and a field sheet indicating field number assigned to that specimen. Ethyl acetate is considered harmless to humans and yet is effective for killing ectoparasites (Fowler 1984).

After fumigating the bird for 15 to 20 minutes, carefully remove the bird from the plastic storage bag over a large sheet of clean white paper. In the field we typically use a lunch tray covered with a large sheet made by taping together two pieces of 8.5″ × 11″ white paper. In windy conditions it is a good idea to use a cardboard box or other windbreak to block the wind. Also, one can tape the paper to the lunch tray to keep the wind from blowing the sheet away, which could result in the loss of ectoparasites. Before ruffling the bird's feathers to dislodge and remove ectoparasites, always check the inside of the plastic storage bag for parasites. If any ectoparasites have fallen off of the host inside the bag, use a paint brush wetted with absolute ethanol to pick up the ectoparasites and place them in a new vial filled with absolute ethanol (do not use denatured ethanol). Hold the bird with one hand and use the other hand and fingers to ruffle all of the bird's feather tracts. Start with the wings, including the primaries and coverts, and then while holding the legs with one hand you can ruffle the feathers of the belly, back, and head. Then hold the head and/or beak (if the bird has a large bill) and "beat" the bird to loosen attached ectoparasites. For small birds one can also hold them between two cupped hands and shake them up and down like

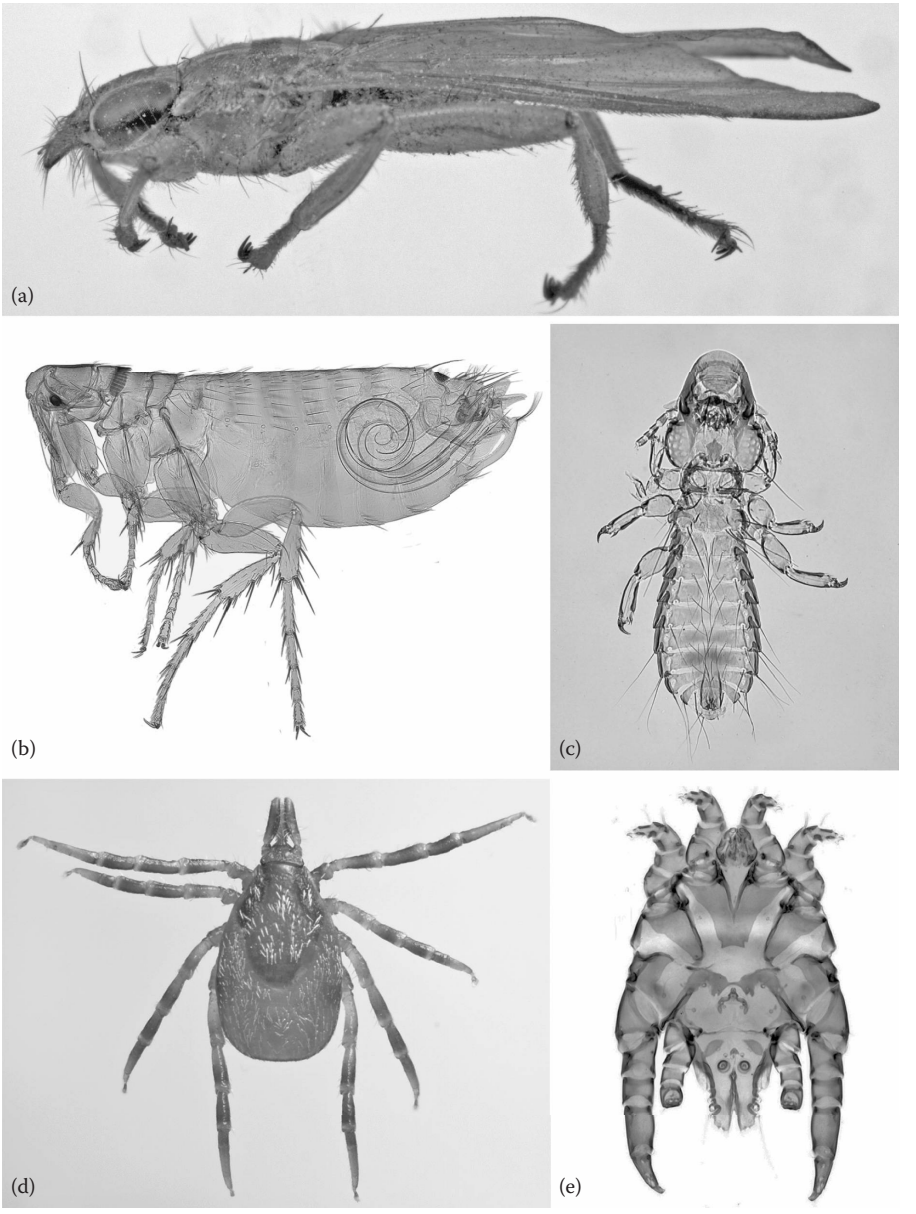

Figure 10.6. Representative images of common ectoparasite groups found on birds. (a) Hippoboscid fly: *Icosta Americana* ex *Accipiter cooperi* (photo by Jason Weintraub). (b) Flea: *Ceratophyllus altus* ex *Campephilus magellanicus* (photo by Michael W. Hastriter). (c) Chewing Louse: *Cotingacola lutzae* ex *Laniocera hyppopyrra* (photo by Michel P. Valim). (d) Tick: *Ixodes brunneus* (photo by Lorenza Beati). (e) Feather mite: *Anomothrix machadoi* ex *Buceros leadbeateri* (photo by Fabio Akashi Hernandes).

dice. This also helps to loosen strongly attached ectoparasites. Furthermore, ectoparasites such as feather lice (Phthiraptera: Ischnocera) can include four ecomorphs that are specialized on different regions of the avian host's body, including the head, wing, body, and generalist ecomorphs (Johnson et al. 2012). Be sure to cover all of the body carefully to thoroughly sample these different ectoparasites.

Pick up all ectoparasites that fall off of the host onto the paper, using the tip of a fine paintbrush moistened with absolute ethanol. Place these parasites into a vial of absolute ethanol. It is best not to use a forceps to pick up ectoparasites because

this may damage morphological features on the specimens. Although many previous papers have suggested using 70% ethanol for preservation of ectoparasites (e.g., Clayton and Walther 1997), we have found that absolute ethanol is best because it preserves both morphology and DNA of the specimen; specimens stored in 70% ethanol will very quickly be useless for DNA extraction. However, if absolute ethanol is unavailable, 95% ethanol can be used in its place for collection and storage of ectoparasites. Place a label made with archival acid-free cotton fiber paper and written using an indelible Pigma Micron pen inside the vial. The label should contain the host taxon name, field collecting number, date of collection, collecting locality, and name of parasite collector. Be sure to note information on the ectoparasite collecting event in the field notes catalog. It is also important to note negative collecting events (when no parasites are found), as these data will allow one to calculate prevalence and intensity of parasitism. After picking up the parasites, continue with several more bouts of ruffling until no parasites fall off of the host. Before moving on to the next host specimen, clean the collecting surface and inspect your hands to be sure there are no contaminant ectoparasites on them.

The ethyl acetate fumigation with postmortem ruffling method outlined earlier will collect most lice, ticks, fleas, hippoboscid flies, and external mites (Figure 10.6). However, this method is not appropriate for quantification for all of these parasites. For permanent ectoparasites, such as lice, which live their entire life cycle on the host, this postmortem ruffling method is quantitative only when conducted to a point of diminishing returns (Walther and Clayton 1997, Clayton and Drown 2001). Moreover, this method is not suitable for quantitatively sampling ectoparasites that live inside the throat pouch, nasal cavities, feather quills, and under the skin. To thoroughly sample avian feather mites, one should visually search through the plumage using a stereomicroscope (although ruffling will allow the collection of some mites). This also allows one to note the locations where each mite taxon is found. For the subset of feather mites that inhabit the wings, one can hold the flight feathers up to the light and look for mites inserted between the feather barbs. One can then use the handle of the paintbrush to disturb and "unzip" the barbs of these feathers so that the mites fall onto the collecting paper. Other mites, such as nasal mites, require flushing the nares with water into a gallon jar, then pouring through a #200 sieve to filter out the mites. Other quantitative methods are available, such as body washing, which removes an even larger fraction of ectoparasites than postmortem ruffling (Clayton and Drown 2001). However, this method is not practical for field survey situations but is useful for smaller-scale studies when specimens can be processed in the lab (e.g., Koop and Clayton 2013). Sometimes embedded ticks do not fall off the host after fumigation. In this case, use a forceps to grab the tick as close to the skin as possible to dislodge it without damaging its mouthparts.

After ruffling, the ectoparasite collector will pass the host specimen on to a bird skinner who will prepare the bird specimen and gently necropsy the carcass to gather standard internal organ data. The bird skinner then will pass the carcass on to the endoparasite collector for further dissection and collection of endoparasites. The bird skinner can either sample liver and heart tissues at this time or can pass labeled tubes to the endoparasitologist to collect these tissues.

Preparation and Curation of Ectoparasite Specimens

After returning from the field, individual vials of ectoparasite specimens can be examined to determine and quantify contents. We examine specimens in a glass dish filled with absolute ethanol and use a stereomicroscope to observe specimens and manipulate them with a paintbrush and/or bent syringe needle. Sometimes we use a glass bulb pipet to return specimens to the original vial. Always be sure that a pipet is clean before reusing it. Specimen preparation methods for each ectoparasite group are taxon-specific and can be used to produce slides for morphological examination and for slides of voucher specimens, from which DNA has been extracted. For morphological examination and vouchering for molecular projects, lice are mounted in Canada balsam using a clearing and slide mounting technique described by Palma (1978), whereas mites are mounted in Hoyer's medium (Baker and Wharton 1952). For DNA extraction of lice we typically use a sterilized syringe needle to make a cut between the head and the thorax or between the thorax and the abdomen of the louse depending on the taxon (Valim and Weckstein 2011) and then place this specimen into the digestion buffer provided

in the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). We then allow the louse to digest over two nights and then follow the manufacturer's directions. In pipetting the liquid from the digestion to the Qiagen filter, we are careful to leave the louse in the original digestion tube. We then add 70% ethanol to the tube to preserve the louse until we begin the slide mounting process. Depending on the size of the louse we elute the DNA off the filter with 50 to 100 µl of buffer AE. We give each louse a unique identifier that includes abbreviations for the louse taxonomic name, host alpha taxonomic name, the date of extraction, and the tube number in that batch of extractions. Rather than wait a long time after DNA extraction, it is best to start clearing and slide mounting vouchers as soon as possible. Other ectoparasites, such as hippoboscid flies, can be kept in ethanol, pinned, or slide mounted in Balsam depending on the size of the fly.

SAMPLING PROTOCOLS FOR THE STUDY OF ENDOPARASITES

Birds can harbor an astounding diversity of parasitic worms from all major groups of helminths (except monogeneans), namely, the cestodes (Eucestoda), digeneans (Digenea), nematodes (Nematoda), and acanthocephalans (Acanthocephala; Figure 10.7). Birds can be parasitized by both adult and larval stages of various parasitic worms, although birds in general have fewer larval stages of helminths in comparison with other major vertebrate groups because they rarely serve as intermediate or paratenic hosts of helminths. Helminths can be found in virtually every part of the bird's body. Although the majority is parasitic in the gastrointestinal tract (GIT), including somewhat unusual sites such as cloaca, inside of the crop, or under the lining of the gizzard, many parasitize other organs, such as the liver and gall bladder, kidneys, bursa of Fabricius, trachea, eyes, and mouth cavity (Atkinson et al. 2008). Adult filarial nematodes can be found in the body cavity, under the skin, on the brain, on the heart, and inside bones. Finally, blood flukes and larval stages of filarial nematodes (microfilariae) may reside in both the venous and arterial sides of the circulatory system. Thus, a complete helminthological examination of an avian host can be a very time-consuming process, especially when it involves larger birds. It is extremely difficult to write a unified procedure for all birds due to the great diversity of avian host sizes, anatomical peculiarities, parasite localization, and parasite loads. For example, waterfowl and other aquatic birds usually—although not always—host greater diversity of parasitic worms than terrestrial birds. The dissection protocol in the next section focuses primarily on helminth recovery from the GIT and associated organs, where the majority of parasitic worms are expected to be found. Illustrations and descriptions of bird anatomy can be found in any general ornithology or vertebrate anatomy textbook, and are also readily available through numerous online resources.

Examination of Gastrointestinal Tract and Other Organs for Endoparasites

Birds are most commonly examined for endoparasites as a part of broader ornithological studies that involve the collection of voucher specimens (e.g., study skins and skeletons) for deposition in museum collections. In such cases, a parasitologist typically receives a body or organs of an already euthanized and skinned bird, which they may proceed to dissect.

Dissection techniques may vary. When the host body is received, you should first carefully examine the exterior of the carcass (particularly around the neck) for the presence of filariid nematodes, which should be easily visible. Each avian carcass should be placed in a dissecting tray of appropriate size. In a comprehensive sampling protocol, such as the one recommended in this chapter, an incision already should have been made to access the body cavity for tissue sampling and sexing of the host. However, if the body is not already opened by the host voucher preparator (e.g., in cases where birds have been donated by hunters), then you will need to open the carcass by making an incision on the abdominal side of the body. The incision should be made with scalpel or scissors along the midline of the body on its abdominal side, approximately from the level of sternum to the level close to cloaca, but not reaching the cloaca. When not preserving the skeleton, one can cut through the rib cage to provide easier access to the organs located in the upper part of the body (esophagus, trachea, heart, air sacs). When making any cuts be careful to not cut the GIT, as this will cause gut contents (and possibly helminths) to spill into the body cavity. This may also happen

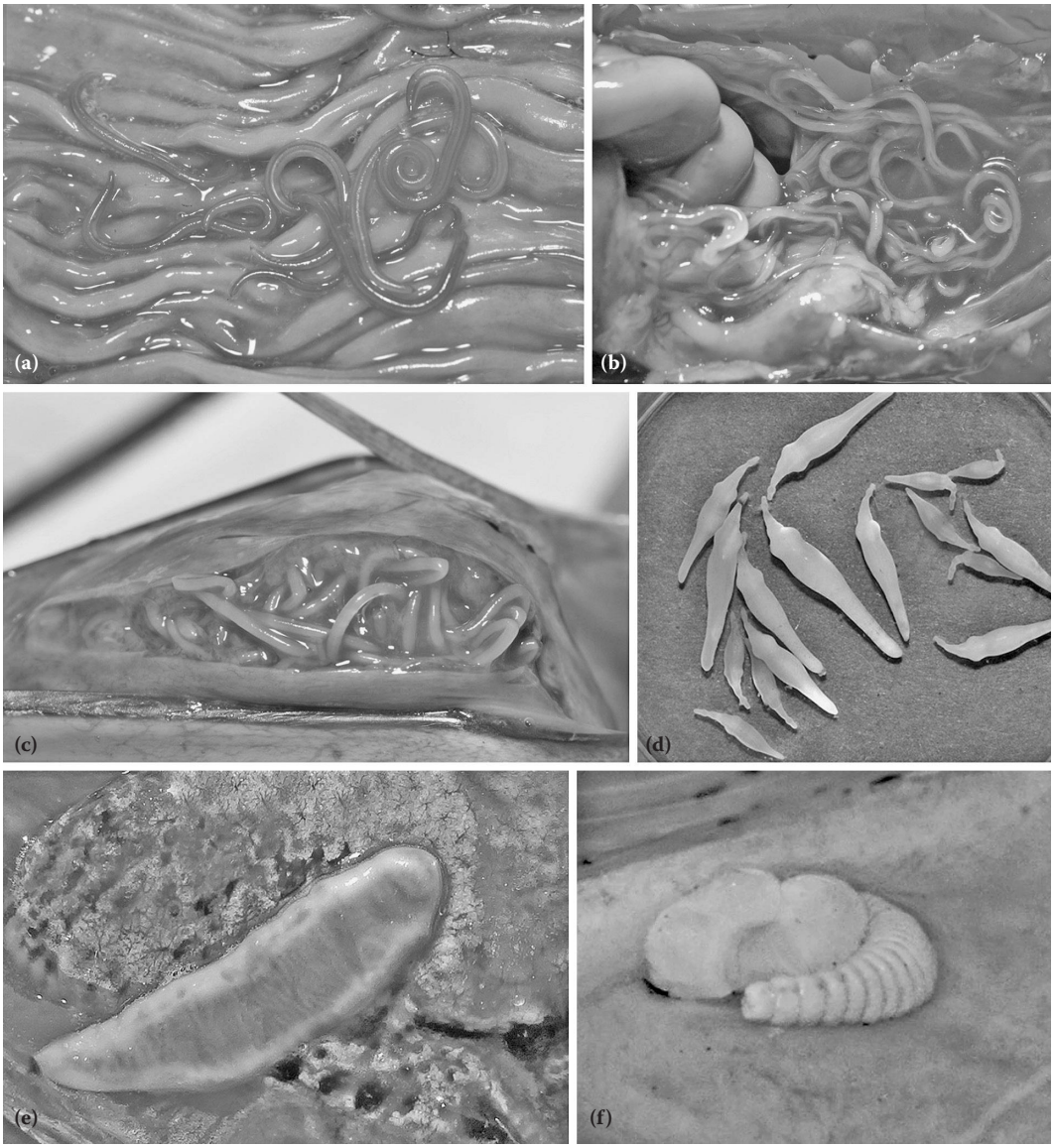

Figure 10.7. Examples of different groups of helminths inhabiting various sites in bird bodies. (a) Spirurud nematodes in stomach of Anhinga *Anhinga anhinga*. (b) Filariid nematodes in body cavity of Barn Swallow *Hirundo rustica*. (c) Dracunculid nematode *Avioserpens* sp. under skin of the chin area of Little Egret *Egretta garzetta*. (d) Acanthocephalans obtained from Northern Shoveler *Anas clypeata*. (e) Hymenolepidid cestode *Cloacotaenia megalops* attached to the wall of cloaca of Northern Shoveler *A. clypeata*. (f) Cyclocoeliid digenean in body cavity (on the lung) of Eurasian Coot *Fulica atra*.

if a bird was shot and the GIT is damaged. As with the exterior of the carcass, you should inspect the interior of the body cavity for visible filariid nematodes once an incision has been made. If blood flukes (Schistosomatidae) are among the targeted parasites, use citrated saline solution (which can be prepared by dissolving 5 g of noniodized table salt and 3 g of sodium citrate $C_6H_5Na_3O_7$ in 1 L of water) throughout this procedure. Make sure to pour some citrated saline into the bird body cavity as soon as you open it. If blood flukes are not a target, use regular saline throughout. Any helminths discovered should be kept alive in saline until fixation.

Remove the complete GIT by carefully cutting connective tissues holding it in place. Then cut

the mesenteries that hold intestinal coils together. When separating the intestine from the liver do not cut the gall bladder. Cut through skin surrounding the cloaca to keep it intact. In young birds, the bursa of Fabricius may be found on the side of the cloaca. It is best to keep the cloaca and bursa of Fabricius together until examination. Move the entire GIT into a tray of appropriate size (e.g., a large glass petri dish for small passerine birds, or a large glass baking dish for ducks or other large bodied birds). At this point, different parts of GIT (esophagus, stomach, small intestine, ceca, rectum, cloaca, bursa of Fabricius, etc.) can be separated for subsequent examination. Remove the liver and put it in a separate dish with saline. The spleen and pancreas very rarely contain parasites, although digenean infections can be encountered in these organs. Carefully remove the kidneys. This can be done by pulling one end of a kidney upward using a forceps of appropriate size and cutting underneath with scissors. Place kidneys into a separate dish with saline. Remove the trachea and place it in a dish with saline. The trachea very rarely serves as a site for helminths, but large digeneans such as *Orchipedum* in cranes and pathogenic nematodes *Syngamus trachea* in galliforms can be found there.

Disrupt air sacs with a gloved hand or using scissors. Carefully rinse the entire body cavity with citrated or regular saline, and pour it from the body cavity into the pan, and then from the pan into a beaker of appropriate size. Allow the contents to settle. This process, called sedimentation, allows endoparasites and other solids to settle to the bottom and the bloody mixture in the supernatant to be discarded so that parasites may be observed and collected for fixation. Once sedimentation is mostly completed, discard the supernatant into another container by carefully pouring it off. Be sure to pour the supernatant slowly to avoid loss of the materials at the bottom of the beaker, then add fresh saline to the sediment. Shake or stir. Repeat the procedure until the supernatant is reasonably clear. Pour small portions of sediment into a petri dish and examine under a stereomicroscope. Although some digeneans can be large, such as members of the Cyclocoeliidae, others, such as those that fall out of damaged intestine or kidneys, may be much smaller. Blood flukes or their fragments, for example, may be extremely small and transparent. Helminths should be transferred using pipettes with orifices of different sizes or lifted with curved forceps, curved needles, or similar instruments. It is important to avoid grabbing and holding any helminths using forceps, with the exception of large nematodes and acanthocephalans, which can be taken and transferred using soft forceps. Handling helminths with forceps almost invariably leads to their damage or destruction.

Examination of the intestine usually takes longer than other organs. The order of organ examination depends on the priorities of your study. We usually examine liver and kidneys first. In small birds, the gall bladder may be studied without separation. In larger birds, however, it is best to separate the gall bladder from the liver and cut it open for examination in a separate small petri dish. Liver and kidneys need to be torn into small pieces, which can be done using scissors or tweezers (especially in the case of very small birds). However, we prefer to gently break apart liver and kidneys with gloved fingers, which preserves ducts for examination and careful dissection of parasites, and reduces the probability of parasites being cut or damaged. Some dicrocoeliid digeneans from the liver (e.g., *Brachylecithum*, *Lutztrema*) and members of the family Eucotylidae from the kidneys can be tightly packed in the ducts and may not be easy to recover. The disrupted liver and kidneys of small birds can be examined immediately under a stereomicroscope. In case of larger birds (e.g., aquatic species), process the liver and kidney tissues using the same sedimentation method as described for the body wash: pour the disrupted liver or kidney tissues into a jar or a bottle with a lid, this time shaking the bottle, then pour the liquid into a beaker, allow for sedimentation to clear the supernatant, and retain the solids at the bottom. Repeatedly add new clean saline and carefully pour off until the supernatant is clear. Once clear, the solid contents at the bottom of the beaker can be examined. There is no need to shake tissues more than once. Examine the sediment in small portions under a stereomicroscope. Besides readily visible digeneans (e.g., Dicrocoeliidae and Opisthorchiidae in liver, Eucotylidae and Renicolidae in kidneys), the liver and kidneys are also important target organs to search for blood flukes. They can be extremely small, transparent, and are frequently fragmented, thus requiring particular attention during sediment screening.

The esophagus can be opened with scissors longitudinally. Some helminths, such as larger nematodes, can be readily seen and removed without optics, but the lumen and walls of the esophagus need to be examined under a stereomicroscope. After examination, you can compress the esophagus wall between two pieces of glass (size and thickness vary depending on the size of the bird), because some nematodes can be located in the wall and can be seen only under compression.

The stomach may contain representatives of several nematode families, digeneans, and even cestodes. The proventriculus and gizzard can be separated before examination. Stomach contents need to be removed and examined for the presence of helminths, nematodes in particular. Upon preliminary examination, the proventriculus wall can be scraped with the edge of a microscope slide and the scraped material examined under the stereomicroscope. Nematodes, digeneans, and cestodes may be found under the lining of the gizzard. In small birds, the gizzard wall lining can be easily peeled using forceps. In larger birds, especially large aquatic birds, peeling the gizzard wall can be more difficult, usually resulting in multiple fragments, and thus should be done in saline. After all of the lining is removed and rinsed, the liquid should be examined for helminths. The tapeworm genus *Gastrotaenia*, which are uniquely parasitic under the gizzard wall lining in anseriform birds, are small and easily mistaken for nematodes.

For the following steps, using scissors with rounded/blunt/balled ends, or at least on one end, is strongly recommended (Figure 10.8a,b). The duodenum and small intestine (Figure 10.8c,d) typically contain the highest diversity and numbers of helminths. If blood flukes are among the targeted taxa, then examine the mesenteric veins and veins of the intestinal wall and cloaca for these parasites before opening the intestine or cloaca. Next, one can remove excessive tissue (e.g., fat and connective tissue) around the intestine and open it with a longitudinal incision using scissors while holding the end of the intestine with tweezers/forceps (Figure 10.8e). For easier detection of tapeworm strobilae, it is best to begin the incision at the posterior end (Figure 10.8f). If a tapeworm is detected, the subsequent dissection can give more attention to find the scolex (or scoleces in case of numerous cestodes) buried in the mucous layer of the intestinal wall. After the entire intestine is open, it needs to be inspected for any other helminths readily visible by naked eye or under a stereomicroscope (Figure 10.8g). Acanthocephalans may be deeply embedded in the intestinal wall and need to be carefully removed. Any other visible helminths also should be removed and placed in a petri dish with saline (Figure 10.8h). In some cases, this is not feasible due to a very high number of helminths, especially if they are very small. To dislodge embedded helminths, scrape the intestinal wall with a side of a microscope slide under a shallow layer of saline solution. It is best to secure the end of the intestine with forceps, and it is essential to press the slide with sufficient force to scrape the intestinal wall deep enough to not break helminths that are deeply embedded in the layer of mucus. If the glass only slides on the surface of the mucus, then it is likely that some worms will be broken while others will remain attached to the intestinal wall in the mucus. Following this step, pour the saline with scrapings into a cylinder or bottle with a screw cap, shake vigorously, and empty into a tall beaker (a water bottle with the top cut can be used in the field) and allow to settle for about 2 minutes, depending on the density of the mix, before pouring off the supernatant. Sedimentation should follow the same basic procedure previously outlined. When the supernatant is clear the sediment should be examined under a stereomicroscope or can be fixed with ethanol for subsequent examination in the lab (for a similar procedure, see Justine et al. 2012).

Fixation of Helminths

The choice of fixative for helminth fixation depends on the purpose and future use of the material. In cases when numerous individuals of the same species of helminths are available and time permits, it is always a good idea to use several fixatives, each optimized for a different downstream purpose. For example, morphological light microscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), histology, immunology, and molecular methods each require a different fixative. Some of these downstream procedures, such as TEM and immunological/immunohistochemical

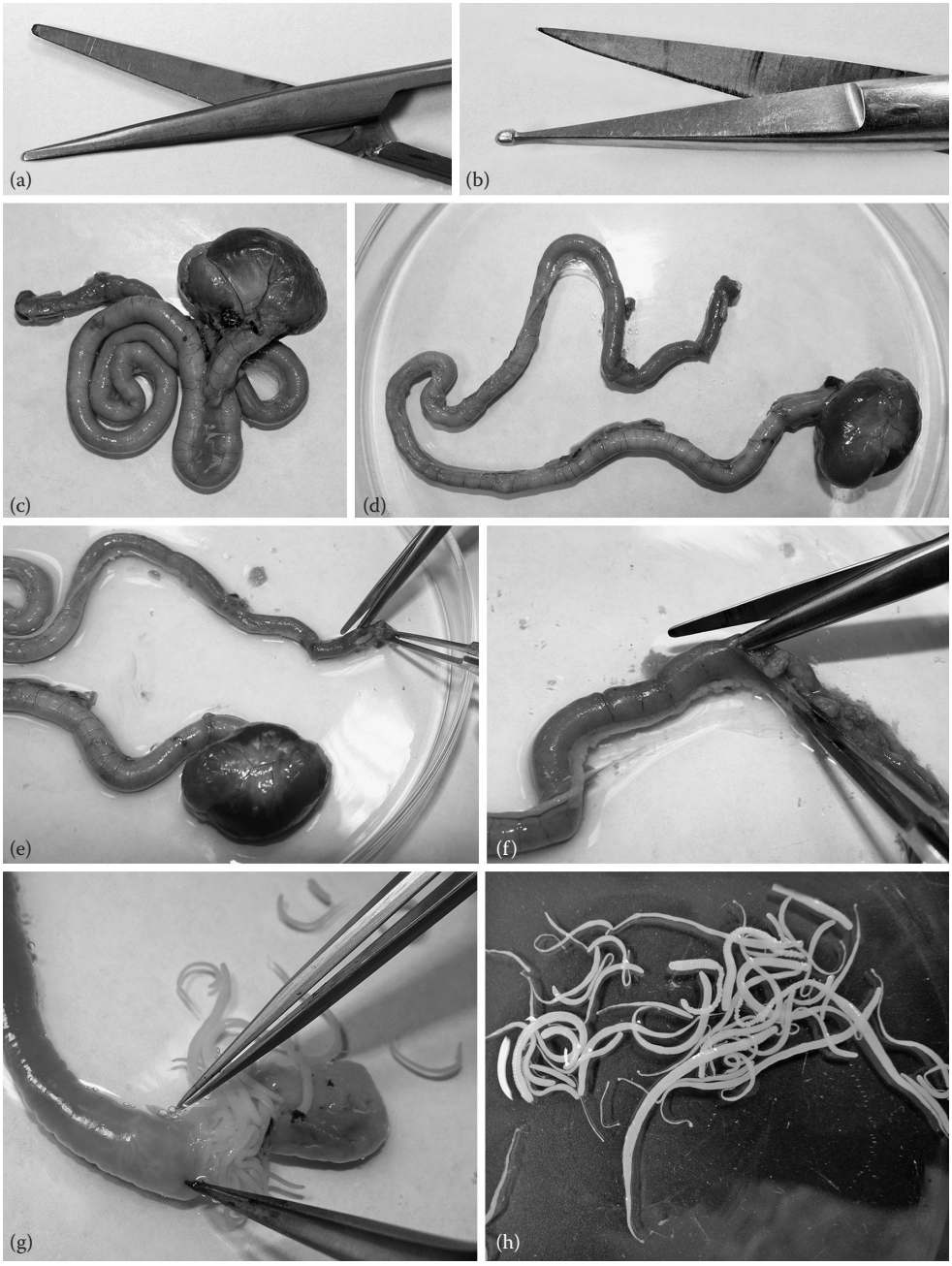

Figure 10.8. Dissection of the intestine of a small bird (American Robin, *Turdus migratorius*). (a, b) Recommended scissors with rounded or balled (so-called artery scissors) ends. (c) GIT removed from bird (esophagus was separated). (d) Straightened intestine with mesentery cut and fat removed. (e, f) Opening of the intestine with scissors starting from posterior end. (g) Gentle tearing of intestine using fine forceps after tapeworms were discovered. (h) Cestodes removed from intestine in saline.

studies, require specialized fixatives, whereas others, such as morphology, SEM, and basic molecular analysis not involving next-generation sequencing (NGS) methods, can use a common fixative. Seventy-percent ethanol is a fixative of choice but with some limitations. Some authors prefer fixation of specimens for future staining with hot (steaming but not boiling) 10% formalin (i.e., 4% solution of formaldehyde) with subsequent storage in formalin. Others fix specimens in formalin with subsequent transfer to 70% ethanol for long-term storage. We have not noticed a significant difference in side-by side comparisons of specimens simultaneously fixed with either fixative (and stained with the same stain). However, the use of heated formalin may have a negative impact on the collector's health due to inhalation of toxic vapors. Furthermore, formalin hinders DNA extraction and subsequent molecular analyses. This is particularly important when considering that morphologically similar ("cryptic") species of helminths are not readily distinguishable in the field and may be present in samples. We therefore advocate the use of 70% ethanol as the single universal fixative equally suitable for "routine" morphological and molecular studies. It is also less hazardous and has fewer transportation restrictions than formalin.

We would like to emphasize that specimens fixed in ethanol (70% or 95%) need to be placed in a freezer or at least a refrigerator as soon as possible and stored long term in a freezer. In the author's lab (V. V. Tkach), 80% ethanol is usually used as the starting concentration in the field because, during pipette specimen transfer to the storage vials, some fluid (water or saline) is inevitably added to the ethanol, which dilutes it to ~70%. Thus by using 80% ethanol we ensure that concentration does not decrease significantly (which would result in poorly preserved specimens). It is recommended that you change ethanol once after initial fixation to ensure sufficient concentration, though this is not always feasible during field collecting trips due to the time and material limitations. As a general rule, for NGS and transcriptomic applications, freezing in liquid nitrogen is the gold standard for field preservation, followed by 95% ethanol for NGS, and RNA*later* (Sigma-Aldrich, St. Louis, Missouri) for studies targeting RNA. Each group of helminths needs to be fixed using a slightly different protocol to properly preserve the morphological features of interest. The following are our recommendations for fixation of live worms using 70% ethanol.

Flatworms (digeneans and small- to medium-sized cestodes) can be heat killed with hot water. Remove most of the saline from a petri dish leaving only a very small amount to cover worms to prevent even momentary desiccation. Pour hot water (steaming, not boiling) onto the worms, and stir the water using propulsion by pipette. Add ambient temperature water immediately to prevent overheating and then transfer the worms into vials with 70% ethanol (again, for practical reasons we use 80%) as soon as possible. In field conditions, a good quality thermos can be used to keep water hot for some period of time rather than reheating it every time one needs to heat kill specimens. Change hot water as needed. Alternatively, flatworms can be pipetted into a petri dish or a small beaker with hot saline (see Cribb and Bray 2010) with subsequent transfer to ethanol. In the case of very thick-bodied digeneans, a subsample can be fixed in ethanol using slight pressure with a cover slip or slide (depending on the worm size). Such specimens can provide a better view of the organization of internal organs. However, specimens fixed in this manner may be distorted and should not be used to make measurements. Tapeworms should never be fixed under pressure. Very large tapeworms usually contract and become less suitable or completely unusable for morphological analysis if the aforementioned heat-killing method is used. Thus, very large tapeworms may be killed and relaxed at the same time by moving them between a petri dish with water (ambient temperature) and a dish with ethanol of weak concentration (10%–15%) using a curved needle or curved tweezers to hold the tapeworm from underneath. Scoleces of large cestodes with armed rostellum can be fixed separately before the strobila is relaxed to avoid loss of rostellar hooks. When the tapeworms die they can be fixed in ethanol. We usually pre-fix tapeworms still in a petri dish before transferring them to a vial of appropriate size. The volume of ethanol in the vial should be at least 4 to 5 times greater than the volume of tissue. Thus, with larger

tapeworms, 50 mL falcon tubes may be the preferred storage container rather than vials.

Larger nematodes with a thick cuticle can be heat-killed following the general procedure outlined earlier. However, hot saline has to be used rather than water to prevent nematode bodies from rupturing due to the difference of osmotic pressure. Instead of hot saline being poured onto nematodes, the petri dish containing nematodes already in saline can be heated using an alcohol burner or lighter until the nematodes die. Heat the saline only to the point that it begins steaming and no further. The nematode cuticle may shrink somewhat in ethanol, even if it is only 70% concentration. Higher concentrations of ethanol may distort nematodes irreversibly and are certainly not recommended for specimens to be used for future morphological examination. Neutral buffered 10% formalin can be used for fixation and does not negatively affect morphology, but we tend to not use it for the aforementioned reasons.

Small nematodes with thin cuticle can be fixed with hot saline or hot 70% ethanol. In the latter case they are simultaneously killed and relaxed. One must exercise caution to prevent ethanol from catching on fire during heating.

For an adequate morphological study of acanthocephalans, the proboscis should be fully everted. This is rarely achieved by heat killing. Leaving them to die in water until the probosci are everted usually produces the best results. When possible, a dish containing water and acanthocephalans should be kept at low temperature (e.g., in a refrigerator), but even at ambient temperature (e.g., in a field camp) the desired result is usually achieved. Then acanthocephalans can be transferred into ethanol.

All vials should have internal labels. Writing information with a marker on the outside of the vial is not sufficient and is likely to result in the loss of data, which may render the specimens useless. Even vapors of ethanol inside a vial storage box may dissolve ink on the outside of vials. Therefore, labels to be placed inside of specimen vials need to be made of a paper that resists prolonged soaking in fixative without deterioration (archival acid free cotton fiber paper is best, but there are other alternatives), and should be written by hand or printed using either pencil or alcohol-proof ink or alcohol-resistant printer ink. In the latter case, the ink needs to be tested prior to use, otherwise there is a risk of losing label information. Ethanol should fill almost all of the remaining space inside the vial, leaving just a very small space for potential expansion at higher temperature. Leaving too much air in vials may result in dried specimens during the transportation of the vial boxes.

Preparing Endoparasites for Morphological Study

Morphoanatomy of flatworms (digeneans and cestodes) is usually studied on permanent total microslide mounts. Parasitic nematodes are usually studied on temporary mounts and acanthocephalans can be studied on either permanent total mounts (mostly to study internal organs) or temporary mounts (mostly to study the proboscis armature and egg structure). There are a plethora of recipes for stains that have been used for trematode and cestode total mounts over the last hundred years. They can be found in numerous manuals and special publications (e.g., Dubinina 1971, Ivashkin et al. 1971, Pritchard and Kruse 1982, Georgiev et al. 1986). We refrain here from a discussion of advantages or disadvantages of one or another staining method. Instead, we provide only two stains and corresponding protocols that have been successfully used in Tkach's laboratory for a great diversity of parasitic flatworms (see Boxes 10.2 and 10.3). Iron acetocarmine, Gill's haematoxylin, and Delafield's haematoxylin are useful alternatives that can be found in the aforementioned references. It is important to realize that there is no one-size-fits-all staining procedure for every kind of specimen, and the amount of time and stain concentration may reasonably vary from taxon to taxon of flatworms. Staining and mounting is as much an art as it is science. Tkach's lab usually uses staining protocols with alum carmine (after Dubinina 1971, with minor modifications) and Mayer's haematoxylin (a somewhat modified protocol used in T. Cribb's laboratory; e.g., Miller et al. 2010). Both stains are water based and require specimens to be rinsed in water prior to staining.

Box 10.2 Alum Carmine Staining Protocol for Endoparasites

1. Rinse worms in distilled water. Rinse time depends on the size of the specimens. Water may need to be changed once for larger specimens. When transferred from ethanol to water, specimens will float. Sink them by pipetting water onto them or by using a soft tool such as a paintbrush.
2. Transfer specimens to stain. The stock solution of the stain can be diluted with distilled water immediately prior to staining. The level of dilution is flexible, but a stain that is roughly 2× diluted usually works well. Somewhat longer staining time with more diluted carmine usually produces better results, but it depends on the group of parasites, fixation, and so forth. Test stain a few specimens of lesser value and you will know what works best. Staining time can be from a few minutes for small specimens with concentrated carmine to more than 30 minutes for large worms using more diluted stain.
3. Transfer specimens to water to rinse off the stain.
4. Destain in acid alcohol (0.5%–1% solution of HCl in 70% ethanol) while observing the specimen under a dissecting scope. The body filling tissue (parenchyma) should be generally free of stain, but enough stain should remain to color the internal organs. Large specimens with thick tegument may not be transparent enough for good assessment of coloration. In these cases one has to rely on experience. Replenish the acid alcohol if it becomes too pink. Destaining may take only seconds in some cases, therefore we recommend using 0.5% HCl solution in ethanol to avoid rapid destaining.
5. Transfer specimens to water to rinse off the destaining solution. At this point specimens can be straightened if needed. This can be usually achieved by stretching specimens on a piece of paper while keeping them under a thin layer of water, then add 70% ethanol and keep adding ethanol in small portions (to keep specimen wet at all times) until the specimen is hardened and can be transferred into a beaker for further dehydration.
6. Dehydration. Specimens need to be moved through a series of ethanols of ascending concentration. Ethanols at 50%, 70%, 80%, 90%, and 100% are recommended (95% can be added between 90% and 100%). To ensure complete dehydration, an additional change of 100% ethanol is recommended. Specimens can be moved through a series of beakers or may stay in the same beaker while ethanol is changed. Either way, make sure that specimens are not exposed to air at any point during the procedure to avoid an immediate desiccation and loss of the specimens. Time in each ethanol depends on the size of specimens; 30 minutes in each grade is usually sufficient for small specimens up to 3 or 4 mm in length. Longer times are recommended for larger/thicker specimens. An hour is recommended in 100% ethanol.
7. Clearing. After water has been removed from the specimens by dehydration, they are transferred to a clearing agent (clove oil [eugenol] is recommended). The clearing agent renders the parasite transparent and is miscible with the mounting medium of choice.
8. Mounting. We strongly recommend damar gum as the embedding medium. It is sold by many suppliers and is clear, cheap, relatively fast drying (much faster than Canada balsam), and xylene soluble so the specimen can be remounted if needed. The embedding medium hardens as the solvent evaporates, making a permanent mount of your specimen. To provide a support for specimens we use precut pieces of cover slips placed on both sides of a specimen prior to covering it with cover slip.

Box 10.3 Mayer's Haematoxylin Staining Protocol for Endoparasites (Similar to Alum Carmine Protocol, with a Few Notable Differences)

1. Rinse worms in distilled water as with alum carmine protocol.
2. Transfer specimens to stain. The stock solution of the stain needs to be diluted at least 1:1 with distilled water. Do not use metal instruments when working with haematoxylin. Use only a dedicated pipette to not mix with other chemicals. Somewhat longer staining time with more diluted haematoxylin usually produces better results, but it depends on the group of parasites, fixation, and so forth. Usually staining takes from 15 to 60 minutes.
3. Transfer specimens to water to rinse off the stain.
4. Destain in 1% aqueous solution of HCl while observing the specimen under a dissecting scope. The body filling tissue (parenchyma) should be free of stain, but enough stain should remain to color the internal organs. Large specimens with thick tegument may not be transparent enough for good assessment of coloration. In these cases one has to rely on experience.
5. Transfer specimens to 1% ammonia solution to neutralize destaining process. Coloration will change from red to blue or purple. Use water to rinse off the destaining solution. At this point specimens can be straightened if needed (see Box 10.2).
6. Dehydration. Specimens need to be moved through a series of ethanols of ascending concentration as in Box 10.2.
7. Clearing. After water has been removed from the specimens by dehydration, they are transferred to a clearing agent. In this case either methyl salicylate or clove oil can be used. Methyl salicylate usually produces somewhat more contrasting coloration. Specimens should be first transferred to a methyl salicylate/ethanol mix in 1:1 ratio and then to pure methyl salicylate. See Box 10.2 for directions on using clove oil.
8. Mount specimens as in Box 10.2.

CONCLUSION

The concept of the "extended avian specimen" is strongly embodied by the comprehensive sampling of avian hosts and their symbionts promoted in this chapter. Collection of avian symbiont data has led to many important discoveries in studies of avian disease ecology and conservation (Atkinson and LaPointe 2009, Parker et al. 2011, Samuel et al. 2015), morphology and development (Clayton and Cotgreave 1994, van Dongen et al. 2013), and evolutionary biology (Weckstein 2004, Whiteman and Parker 2005). Such discoveries could not have been made based on the study of host voucher specimens in the absence of symbiont data. Therefore, as technology and sampling methods continue to improve, and as species and their symbionts face ever-increasing threats to their existence, it is crucial for avian biologists to consider not only the birds we are studying, but the plethora of microbes and parasites that are living in and on each bird. By investing in these aspects of the extended specimen, researchers will preserve data that may shed light on many important areas of avian biology, as well as provide data for myriad microbial and parasitic taxa that are relatively poorly studied.

For ornithologists who do not intend to study parasites or pathogens directly, or who do not have the facilities to curate their specimens, many options exist for ensuring that specimens find their way into collections where they will be curated and utilized. We strongly recommend that type and voucher specimens be deposited in museum collections. In the United States, the main collections curating helminth and other endoparasite specimens, and providing loans for studies, are the U.S. National Parasite Collection (now a part of the Smithsonian Institution, Washington, DC), Harold W. Manter Laboratory of Parasitology at the University of Nebraska (Lincoln, NE), and the parasite collection of the Museum of Southwestern Biology (Albuquerque, NM). Most museums with large entomological holdings will

also house collections of arthropod ectoparasites and are appropriate places for depositing this material. There are, of course, a large number of other parasitological collections both in the United States and around the world. We recommend submission of specimens to museums that provide specimen loans for examination. Several publications (e.g., Lichtenfels and Prtichard 1982, Lamothe-Argumedo et al. 2010, Zinovieva et al. 2015) provide useful information on location and scope of taxonomic coverage of the most important helminth museum collections.

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