# ECOLOGICAL MONITORING AND ASSESSMENT NETWORK

# (EMAN) PROTOCOLS FOR MEASURING BIODIVERSITY: PARASITES OF BIRDS

**by**

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# I. INTRODUCTION

The focus and ecological context of this review is on the parasites of Canadian birds and reflects the experience of the author. Although presented from this perspective, many of the issues raised (e.g. permits) and the various techniques and procedures described are broadly applicable regardless of where studies are done. The review describes general techniques used to collect and process endoparasites, ectoparasites and blood parasites in avian hosts. It is intended primarily as a reference for researchers with little experience with parasites and is not necessarily meant to be read in it's entirety.

Collection of parasites is labour intensive, time consuming and requires some expertise. Ideally, parasites should be collected from freshly killed hosts. This inevitably places additional demands on a study and it is difficult for researchers focussed on other aspects of the host's biology to find the time to collect parasites properly. The result is often poor, frequently unidentifiable, specimens and questionable counts. If parasites are to be included as part of a larger study a parasitologist, or someone trained in the appropriate techniques, should be consulted in the planning stages of the project and sufficient time and resources need to be allocated to do the work.

## A. PARASITES AND PARASITES OF BIRDS

Parasitism is an extreme form of a broader phenomenon called symbiosis. In symbiotic associations, one species (the symbiont) lives in physical association with another, larger, species (the host) that provides the physical habitat for the symbiont. Parasites are metabolically dependent on their hosts, hence the association is an obligatory one for the parasite. Parasites have the potential to harm their host (Marquardt and Demaree, 1985; Zelmer, 1998) but it is their ability to evade the host

immune response that distinguishes them from other symbionts (Zelmer, 1998).

Parasites are an integral part of the natural history of animals and are of interest in their own right. They form an important, though usually overlooked, component of the biodiversity of ecosystems. The number of parasite species that exists is unknown. Price (1980) has suggested that there are more parasitic species than free living ones and it is not unusual for birds, particularly those associated with aquatic habitats, to be infected with several species of parasites. Many ectoparasites (which live on the surface of the host) are readily visible and often seen when handling a host. Endoparasites (which live within the host) are only seen on postmortem examination and are seldom encountered by most researchers.

Parasites may affect hosts at the individual, population or community level. At the individual level, parasites can cause disease and death of the host. The effects are usually density dependent and heavy infections are often encountered in dying and dead individuals. However, parasite loads that number in the hundreds or thousands of individuals may also occur in what appear to be otherwise healthy birds.

Parasites may also affect hosts at the population and community levels. They may regulate host populations and influence host community structure (see reviews by Holmes and Price [1986] and Minchella and Scott [1991]) and may affect biodiversity by interfering with processes as diverse as competition, migration, speciation and stability of ecosystems (Combes, 1996). Paradoxically, while parasites may be detrimental to biodiversity in some instances, they may actually preserve it in others (Combes, 1996).

More recently, parasites have become a concern in conservation issues. As contact between otherwise segregated populations or species of hosts increases due to

declining habitat, loss of biodiversity due to parasites becomes more of a threat. Scott (1988), Loye and Carroll (1995) and Holmes (1996) provide detailed discussions of the potential impact of parasites on hosts crowded into declining, and increasingly more fragmented, habitats.

Parasites must continually infect new hosts. The transmission process, or life cycle, may be direct or indirect. In species with a direct life cycle, only one type of host (called the definitive host) occurs. Definitive hosts are those in which the parasite attains sexual maturity (or, by convention, the vertebrate host when one is present in a protozoan life cycle). Parasites
with direct life cycles are transmitted to new hosts by infective stages produced within a definitive host that are passed into the external environment. Although exceptions exist, new hosts generally acquire infections when they ingest the infective stage in contaminated food or water.

In species with indirect life cycles, the infective stages shed from definitive hosts are not directly infective to another definitive host. Instead, they require a period of development in at least one different species of host, called the intermediate host. Within the intermediate host, development progresses to an infective stage that can then be transmitted back to a definitive host. Usually, this is a long-lived stage and often persists for the life of the intermediate host. Except for schistosomes, most parasites with indirect life cycles are transmitted via predation through the food chain or by vectors (blood feeding invertebrates). Parasites that occur in different hosts during their life cycle may have repercussions at different trophic levels within the ecosystem (Marcogliese and Cone, 1997).

Anderson and May (1979) divided parasitic organisms into two broad categories

that transcend taxonomic boundaries. Microparasites (viruses, rickettsias, bacteria, protozoans and fungi) are small organisms that increase in number by multiplying within the definitive host. Macroparasites, which include the helminths (i.e. members of the Phylum Platyhelminthes, Nematoda and Acanthocephala) and arthropods, are larger and populations increase within the definitive host by recruitment of new individuals rather than by multiplication of existing ones.

## A.1. PARASITES OF BIRDS

The parasites of birds have received extensive study and Rausch's (1983) review is an excellent starting point for anyone interested in the topic. The articles in Clayton and Moore (1997) provide a comprehensive treatment of more recent aspects of parasitism in birds and Hudson (1996) has provided a concise overview of the effects of macroparasites and populations of wild hosts, with an emphasis on parasites in birds.

Birds are host to a wide variety of microparasites; however, discussion of this group will be restricted to two phyla of protozoans; the Sarcomastigophora and the Apicomplexa. Viruses, rickettsias, bacteria and fungi also infect birds and detailed information on diseases caused by them can be found in Davis et al. (1971), Wobeser (1981, 1997), Friend (1987) and Nuttall (1997). Gough (1997) provides detailed instruction on collection and identification techniques for these organisms.

Helminths are the predominant macroparasites found in birds. The platyhelminth fauna (flukes and tapeworms) is particularly diverse but many species of roundworms (Phylum Nematoda), thorny-headed worms (Phylum Acanthocephala), ticks, mites, fleas, and lice (Phylum Arthropoda), and a few species of leeches (Phylum Annelida), and pentastomes (Phylum Pentastomida) also infect birds (classification follows Roberts and Janovy, 1996).

There have been numerous studies of bird parasites in Canada and comprehensive lists of the haematozoa (blood parasites) (Bennett et al., 1989), cestodes (waterfowl only) (McLaughlin, 1989), nematodes (Wong et al., 1990) and arthropods (Wheeler and Threlfall, 1989) are available. Similar compilations of the digenean, cestode, and acanthocephalan species that infect birds are needed.

## B. SPECIAL NEEDS

### 1. PERMITS

Most species of birds found in Canada are covered by the Migratory Bird Protection Act and fall under federal jurisdiction; the rest fall under provincial jurisdiction. In either case, it is necessary to obtain the appropriate permits before any collecting is done. Permits to collect migratory species in Canada are issued by the Canadian Wildlife Service, normally from the office in the province or region where the collecting is to occur. Permits for non-migratory species are issued by the appropriate provincial department, usually the Ministry of Natural Resources. Applications for permits should be submitted well in advance of the proposed study because of the time required to review the request. Contact the appropriate agency before filing the application to determine what information is required. At minimum, expect to submit a project proposal, a justification of the species and the numbers requested, an environmental impact statement and a valid animal care certificate for the project.

In Canada, some provinces require special permits to carry a firearm outside of the hunting season. These are usually issued by the Ministry of Natural Resources. Contact the office in the appropriate jurisdiction for further information.

Comparable information regarding permits in the United States is available in Anderson (1999). Anderson also offers a number of useful suggestions for individuals

applying for collection permits in foreign countries. While aimed at American researchers, this information is helpful for anyone applying for a foreign collection permit. This information is available on the internet <http://www.nmnh.si.edu/BIRDNET>. Hard copies are available at nominal cost and can be ordered through the website.

## 2. QUALITY ASSURANCE / QUALITY CONTROL

The goal of any biodiversity study is the accurate identification of the species present and a reliable estimate of their numbers. The accuracy of the identifications will depend on the quality of the specimens available; the accuracy of the numerical estimates will depend upon the thoroughness of the examinations and the sample size.

The collection of parasites for taxonomic work presents special challenges. It involves an examination of the host during which parasites must be recognized, removed, cleaned, and preserved for later study. Endoparasites, particularly those in the gut, deteriorate rapidly following the death of the host and it is essential to remove, clean and preserve these as quickly as possible after the host has been killed in order to obtain specimens in the best possible condition for identification.

Identification of most parasites to the generic level is relatively straightforward. The basic skills can be acquired with practise and keys to the generic level are available for most groups. Unfortunately, there are few keys available to the species level and many of those are out of date. Accordingly, species identifications will often require consultation of original descriptions in the literature. Ideally, someone with experience in the particular taxonomic group should identify the parasites or supervise and verify the results of those who do. Access to identified specimens in reference collections can be invaluable to those with little taxonomic experience in the particular group.

## 3. DATABASE MANAGEMENT

A well kept log book, with information entered as it is obtained, is the foundation of any computerized database. Actual entry and management of the data depends on the nature of the study. Incidental data from a single host or a small number of hosts obtained opportunistically as a result of some form of accidental mortality (e.g. banding mortality) are not handled in the same way as those from larger studies. Regardless, accurate collection data on the host and details of the location of the parasite within the host are essential. Rigorous proof reading is essential when entering data. Always have backup copies (electronic and hard copies).

### *3.1. Host Data*

Include as much information as possible on the individual host. Pertinent data would include the species, age and sex of the individual, the size or weight if applicable, any code number used to identify the host, the collection date, the collection locality and the name of the collector. Identify the collection locality as specifically as possible either by the accepted geographic name, by the distance and direction from a specific locality, or by latitudinal and longitudinal coordinates.

### *3.2. Parasite Data*

During collection of parasites, the site or organ where the parasite was found and the fixative and preservative that was used should be recorded in the log book. Information on any gross pathology seen during examination (bleeding, inflammation, lesions or scar tissue) should also be recorded. Data on food items found in the digestive tract are also important and may prove useful in interpreting parasitological observations.

### *3.3. Additional Data*

Depending on the nature of the study, abiotic and biotic environmental factors associated with the area where the bird was collected (e.g. environmental anomalies such as drought, flood, or time since the event occurred, the presence of other host species, insect emergences, etc.) may be of significance. Any behavioural anomalies (for sick birds) should be recorded.

### *3.4. Data Entry*

Each host is a separate record in the database. All codes and any grouping variable (i.e. any spatial, temporal or biological variable such as locality, month, season, species, sex, age, breeding status, etc.) should be clearly defined. Each species of parasite is considered as a separate variable. The number of each species found in each host (including 0's) should be recorded in the log book and in the data base as soon as they are identified and counted. The information can be stored in spreadsheet format and imported into statistical programs as required for summary and analysis. Proof read the data carefully. Keep backups (electronic and hard copies) and update them immediately when new data are added.

## C. IMPORTANCE OF STANDARDIZED METHODS

Examination of a host for parasites is labour intensive. The number and variety of parasites found will depend in part on their size and numbers, on the size of the host, on the volume of material to examine and on the skill of the observer. This holds regardless of whether one is examining blood smears for haematozoans, feathers for lice or gut contents for helminths. Ideally, all macroparasites should be collected and this is often possible in small hosts or in individuals with light infections, especially if the parasites are large. In most studies however, some specimens will be missed. If possible, it is best to have the same person (or team) perform all examinations, following standardized protocols to ensure that the data from each host are comparable. It is not

possible to count microparasites although methods for estimating the numbers of protozoan infections from samples are available.

# II. AVIAN HOSTS AND THEIR PARASITES

## A. INTRODUCTION

Wild birds are highly mobile and forage in a variety of locations and habitats, increasing the opportunity of exposure to a wide range of parasites. In some instances, this may bring them into contact with domestic species and can result in an exchange of parasites between them. Many species shift diets during the year and most species undergo annual migrations which may have a significant impact on the parasite fauna. Accordingly, local and seasonal movements need to be taken into consideration when planning studies on parasites of birds.

## B. ABIOTIC FACTORS

With the exception of those transmitted by vectors, virtually all parasites have one or more stages that are directly exposed to the external environment and vulnerable to environmental extremes. Dessication, abnormally high temperatures and freezing conditions may be lethal to them.

Abiotic factors may affect parasites indirectly. The physical characteristics of a habitat will influence the animal community present, the complexity of food webs that exist and, ultimately, the species of parasites that are transmitted. In wetlands, conditions such as the extent of the littoral zone, the composition of the substrate, water temperature and depth may affect primary productivity and the presence and distribution of vegetation. This may in influence the local parasite fauna by affecting the distribution and abundance of intermediate hosts. Large scale phemonena, e.g. drought conditions, will reduce the number of invertebrate species present (Jefferies, 1994)

limiting transmission to those parasites whose hosts persist. Conversely, situations where water levels are above normal may produce a dilution effect, reducing contact between hosts and parasites. Marcogliese (submitted) gives an excellent overview of climate change and its potential effects on parasite transmission in aquatic habitats. Similarly, drought or local flooding in terrestrial environments may also affect transmission of parasites, either by affecting the parasite directly or by displacing or eliminating intermediate hosts.

# C. SAMPLING PROCEDURES

## 1. GENERAL CONSIDERATIONS

Birds have been studied extensively for parasites and references to previous studies can be found in the Protozoological Abstracts, Helminthological Abstracts, Abstracts of Entomology, Entomology Abstracts, Review of Medical and Veterinary Entomology, Biological Abstracts, Zoological Record, and the Host Index Catalogue of Medical and Veterinary Parasitology (which ceased publication in 1982). Information from previous studies can be particularly valuable in determining what parasites are likely to be present in a particular host or locality and the frequency with which they are likely to occur.

## 2. HABITAT DESCRIPTION

Detailed descriptions of the physical characteristics of collection sites, of the types of vegetation present and the animal communities associated with them can provide important insights into the interactions between hosts and parasites. These data are also necessary to document any natural or man-made changes and their consequences on parasite populations at a later date (Bykhovskaya-Pavlovskaya, 1964; Marcogliese, submitted).

## 3. SAMPLE SIZE

Data on the parasites from even a single host can provide useful qualitative information on the presence of particular species locally. Larger samples are necessary to estimate frequency of occurrence and population parameters. Different methods of determining sample size, based on presampling data, can be found in Southwood (1978). Unfortunately presampling data are seldom available for parasite studies.

In parasitology, the host is the sampling unit (Holmes and Price, 1986). Thus each the host (and it's parasites) from a particular sampling site or period are considered replicates. Gregory and Blackburn (1991) consider sample sizes of 50 or more hosts sufficient to ensure that all parasite species present in the host population are detected. Post and Millest (1991) examined the problem from a different perspective. They considered a common situation encountered in parasitology; namely, if a particular parasite known to be present in a host species has not been found in a survey, what is the probability that it is present in low frequency but not detected due to sample size? They estimated that the maximum likely frequency of such a species in a negative sample was 13.9%, 3% and 0.6% where the host sample consisted of 20, 100 and 500 individuals, respectively.

When sampling a host population, it quickly becomes evident that some parasites occur frequently, some less frequently but are still common, and some occur infrequently, in small numbers, and form a minute component of the parasite fauna (e.g. Bush and Holmes, 1986; Edwards and Bush, 1989; Bush, 1990; Alexander and McLaughlin, 1997). One of the issues in any biodiversity study is the importance of rare species. Parasites may be rare in a host population for a number of reasons as the following example illustrates. Bush (1990) examined 34 adult willets (*Catoptrophorus semipalmatus*) from four freshwater sites in western Canada (the juvenile component of

the sample is excluded here). Three samples of adult willets from Alberta; Brooks (n=5), Foremost (n=5) and Tilley B (n=19) and one from Manitoba (n= 5) yielded a total of 34 species; 23, 18, 26 and 16 species, respectively. Nine species occurred at all four sites, seven at three, eight at two and 10 at one. All 10 present at one site were found in a single host. The life cycles of six of these 10 species are known and all require marine intermediate hosts, as do two of the eight species found at two sites. These species were remnants of populations from marine wintering areas disappearing due to lack of recruitment. At least three of the remaining six species present in willets at two sites normally infect other birds, primarily anatids. The overwhelming majority of the remainder were helminth species characteristic of willets (or other Charadriiformes) in freshwater habitats and all were detected in at least three of the populations sampled despite the differences in sample size. Based on Bush's study, a sample of 20 individuals should be sufficient to detect the frequent and common species and at least some of the rare species in a host population although some rare species will likely be missed. Consideration must also be given to the type of host being studied. Bykhovskaya-Pavlovskaya (1964) in one of the early attempts to deal with the problem of sample size, suggested that 15 hosts should be sufficient to determine the basic fauna of an aquatic species (consistent with the willet example above) but that larger samples (25-30) are required for upland birds due to different exposure dynamics. The point is that the parasite species characteristic of a particular host in a particular region can be detected with relatively small sample sizes. Some of the rare parasites in a host species will be remnants of populations acquired elsewhere and not part of the local fauna. Others may be parasites that normally infect sympatric host species but only occur occasionally in the host in question.

## 4. QUALITATIVE SAMPLING

Males and females usually have similar parasite loads (Bush, 1990). Unless males and females are spatially segregated or have different feeding habits or diet, sex related differences are unlikely. Young birds tend to acquire the same species as adults, although the magnitude of the infections is often greater. Many birds change diets or foraging areas during the course of a season. This may enhance recruitment of some parasites and restrict or preclude that of others. Most birds undergo annual migration and this may also have a significant impact on the parasite fauna. Parasites of migratory birds fall into four broad categories: species that are ubiquitous (i.e. present in the host year round) and species that are normally present only during the summer or the winter or, briefly, during migration (Dogiel, 1964). Longer lived species acquired in one type of habitat (e.g. coastal areas) may be carried to other habitats and persist for varying lengths of time, despite the fact that local transmission is impossible (e.g. Bush, 1990; Anderson et al., 1996). When comparisons are made between genders, cohorts, habitats or seasons separate samples of appropriate size are necessary.

## 5. TIME FRAME OF SAMPLING

Parasite populations are dynamic and may vary within or between seasons. Collections should be made within the narrowest time frame practical to ensure that samples are homogenous. This can be accomplished if samples are collected from a single site within a period shorter than the life span of the parasites (Janovy et al., 1992).

## 6. FIELD OPERATIONS

### A. SAMPLE COLLECTION - HOSTS

Blood parasites and ectoparasites are usually collected from live hosts. These can be captured by several methods, including mistnets, cannon nets, and a variety of traps (drive traps, nest traps, bait traps and decoy traps) which are effective for certain species under specific conditions. An advantage of live capture is that a number of birds are usually available and these can be aged and sexed and individuals selected as necessary to meet requirements of the sampling protocol.

Alternatively, they can be held and examined later for endoparasites. Birds should be examined as soon as possible to preclude any stress-induced loss of intestinal species. This may vary among species. Ring-billed gulls can be held for several days before helminths begin to appear in the faeces (Levy, 1997). On the other hand, mallard ducks passed helminths within hours of being captured (personal observations) although it is not clear whether this was stress-related or simply reflected a natural turnover of parasite populations.

Birds must be killed humanely prior to necropsy. Information on euthanasia procedures for birds is available in Gullett (1987) or from the Canadian Council on Animal Care, 314-350 Albert Street, Ottawa, Ontario, K1R 1B1. Normally, birds collected for studies on endoparasites are obtained by shotgun. While lead shot remains legal for upland shooting, nontoxic shot must be used near wetlands.

Injured or sick birds encountered in field studies are another source of parasite material. However, permits are still required to legally kill a migratory bird; the fact that it is in distress is irrelevant. While injured birds pose no threat, caution is necessary when

dealing with sick birds. There are a number of serious avian diseases that could be transmitted inadvertently to captive or domestic species (see Davis et al., 1971; Wobeser, 1981, 1997, and Friend, 1987). Principal investigators and field assistants should be familiar with their clinical signs. A useful Wildlife Disease Manual, produced by the Canadian Cooperative Wildlife Health Center (CCHWC), Department of Veterinary Pathology, WCVM, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, S7N 5B4, is available at nominal cost. Unless a condition is of suspected parasite etiology, birds displaying symptoms should be killed, bagged individually and submitted to the nearest veterinary diagnostic laboratory for examination.

Contact the laboratory for specific instructions prior to shipment. The CCWHC maintains a hotline (1-800-567-2003) where information on diseases and the addresses of regional laboratories is available. General guidelines for packing and shipping specimens can be found in Fransen (1987) and in the CCWHC Manual.

**B. SAMPLE COLLECTION - EQUIPMENT AND SUPPLIES**

The equipment and materials required are standard items and are readily available in most laboratories. A list of suppliers is provided in Appendix III. The basic equipment includes compound and dissection microscopes, a laboratory balance, dissecting pans of varying sizes, an assortment of variously sized beakers, culture dishes and Petri dishes, an assortment of glass vials of varying sizes with tight fitting caps (no corks), dissection instruments (regular and small scissors, regular and fine forceps, dissecting needles, scalpels and a pair of bone cutters), Pasteur pipettes and rubber bulbs, a few 10X10 cm pieces of plate glass with polished edges, slides and cover slips (22X22 and 22X50 mm), slide boxes, plastic garbage bags and freezer bags. An electric engraver, available at hardware stores, is particularly useful for marking slides, otherwise use a diamond or carbide engraving pen. Depending on the

study, other equipment or supplies may be required. Lancets, haematocrit tubes, hypodermic syringes and needles are needed for collection of blood parasites; paper bags or other containers for isolating hosts are necessary for the collection of ectoparasites. A supply of specimen labels and data sheets will also be required.

The basic reagents and chemical supplies required are listed in Appendix IV. These include: sodium chloride (for saline solutions), fixatives, 95% and absolute ethanol, blood and histological stains, xylene (or other clearing agent) and mounting media. Commercially prepared solutions and / or ingredients can be obtained from suppliers listed in Appendix III.

Although blood parasites and ectoparasites can be collected in the field, it is much easier to examine hosts for endoparasites in a laboratory. If it is necessary to examine hosts in the field, substitution of disposable aluminum cooking pans for dissection trays, plastic containers for glass ones and apportioning of chemicals into premeasured packets or small concentrated volumes for dilution on site will greatly reduce the weight and volume of equipment. Bush and Holmes (1986) describe a convenient and effective way to quick freeze specimens in the field.

## 7. LABORATORY PROCEDURES

The procedures for collecting, preparing and studying endoparasites, ectoparasites and blood parasites differ to some extent and will be covered separately in Sections 8, 9, and 10. Each section has a brief introduction and is organized as follows:

**1. Collection:** a description of techniques for the removal of the parasites from the host.

**2. Sample Preparation:** a description of **fixation**, **storage, staining** and **mounting** techniques commonly used to prepare the material for study.

**3. Identification:** a list of some of the major taxonomic works useful for identification of specimens to the generic level. This is intended as a starting point, not a definitive listing of taxonomic works. Additional references to taxonomic works on specific parasites and parasite groups can be found in the various abstracting sources listed in Section C.1 and in guides to the biological or zoological literature (e.g. Bell and Rhodes, 1994).

**4. Quantification:** a discussion of the standard methods used to determine or estimate the number of parasites in or on a host.

# 8. ENDOPARASITES

## 8.1. INTRODUCTION

Virtually every organ and internal cavity of birds is infected by some species of endoparasite (Appendix I and II; Doster and Goater, 1997). Coverage of the microparasites will be limited to coccidians (*Eimeria*) and flagellates (*Trichomonas*). The predominant macroparasites are helminths, but a few species of pentastomes and leeches and several species of mites also infect birds. The leeches and mites are covered in Section 9 (ectoparasites).

## 8.2. COLLECTION - MACROPARASITES

Endoparasites are obtained by post mortem examination of the host. In the procedure described below, it is assumed that the entire bird will be examined. However, examinations are seldom this extensive because most studies are limited to a particular group of parasites or to a particular organ or system. This procedure can be

modified as required to meet the needs of any study. There are several steps involved. These include: exposure of the internal organs, removal and separation of the organs, examination of the body cavity and air sacs and examination of the organs.

Before beginning the dissection, inspect the outer surface for lumps or surface lesions. Some helminths (e.g. *Collyriculum* and *Avioserpens)* and bot fly larvae live in subcutaneous sites that open to the exterior through pores or lesions in the skin.

### *8.2.1. Examination Procedures - Exposure of Organs*

1. With the bird on its back, make an incision in the skin extending from the throat to the anus.
2. Reflect the skin laterally to expose the neck, breast and abdomen. Inspect the musculature for parasites and lesions. If the skin is to be examined further, keep the exposed surface moist.
3. Make an incision along the abdominal midline extending from the posterior margin of the breast to the anus. Take care not to damage underlying organs.
4. Cut around the posterior margin of the breast and proceed anteriorly, through the ribs on either side of the breast until it free. Carefully remove it to expose the internal organs.

### *8.2.2. Alternative Procedure*

1. Follow steps 1 to 3 above.
2. Make the abdominal incision from breast to the anus as in (Step 3) above. Continue anteriorly by cutting the breast muscle along one side of the keel to the wishbone area. Retract the muscle to expose the breast bone and cut it lengthwise along the base of keel.

3. Separate the halves to expose the organs.

### *8.3. Examination Procedures - Removal of Organs*

This section describes the removal of internal organs. These should be placed in separate containers and kept moist until examined. Parasites may be encountered in the air sacs and body cavity. They should be removed, placed in separate dishes of water or 0.85% saline until they can be cleaned and fixed.

1. Inspect the space around the heart. This is the interclavicular air sac which is exposed when the breast is removed or opened. Remove any parasites, rinse the air sac and save the water for later examination. Next, examine the surface of the internal organs for parasites. Rinse them and save the water in a separate container.
2. Examine the connective tissue around the trachea and esophagus; filarial nematodes may occur there. Sever the esophagus and trachea from the head just anterior to the glottis and separate them from the connective tissue along the neck. Finally, separate them from each other.
3. Carefully separate the liver, gizzard and intestine from the air sac membranes. Grasp the liver and gizzard and pull backwards. Done properly, the esophagus should slide past the heart leaving the it and the lungs intact.
4. Remove the organs from the body cavity, leaving them attached to the host at the anus. Check for the bursa of Fabricius which is attached to the dorsal surface of the cloaca in young birds. If present, loosen it from the body wall, then detach the organs by cutting around the anus making sure not to damage the cloaca or the bursa.
5. Place the entire digestive tract in water. Shake it to dislodge any parasites that may be on the surface and place it in a clean dish of water. Add the rinse water to the previous wash from the body cavity (Step 1) and allow it to settle.

6. Separate the esophagus, proventriculus, gizzard (or stomach, if present), liver, spleen, pancreas, gallbladder, intestine, caeca, and bursa of Fabricius. Place each in a separate container. Detach the mesenteries from the intestine and save separately.
7. With the visceral organs removed, re-examine the body cavity then rinse and add the wash to the others from the body cavity (Steps 1 and 5). Examine the air sacs, then open each and rinse with water. Add the rinse water to the previous wash from the air sacs (Step 1).
8. Remove the kidneys and ureters, oviduct (if present), heart, lungs and trachea. Place each in a separate dish and cover with water.
9. Remove the head for examination of the mouth, sinuses, eyes and brain. Cover with a wet paper towel to keep it moist. Retain the carcass if parasites or lesions were present in the musculature or if a more complete search for subcutaneous, joint or muscle-inhabiting parasites is to be done.

### *8.4. Examination Procedures - Freezing and / or Fixing of Host Organs and Tissues*

The foregoing assumes that the bird can be examined as soon as it is killed. However, when it is impossible to examine the host within a reasonable period (several hours or more), the only option is to freeze or fix the organs of interest. In either case the bird should be eviscerated as described above and the organs processed as rapidly as possible to minimize post mortem degradation of the specimens. Birds are well insulated so evisceration is essential to ensure rapid freezing - do not freeze the bird intact.

Rapid freezing can be done in liquid nitrogen or on dry ice. See Bush and Holmes (1986) for an effective technique using dry ice and ethanol that can be used in

the laboratory or in the field. Organs can also be frozen in an ultracold freezer (-80$^{o}$ C) or in a regular freezer (-20$^{o}$ C) as a last resort. Frozen specimens are inferior to those fixed fresh but the extent of the damage varies. Cestodes tend to loose rostellar hooks and fragment easily on thawing so extra care is required when the intestine or ceca are examined. Digeneans, nematodes and acanthocephalans are more robust but also suffer some physical damage when frozen. As a rule, frozen specimens do not stain as well as those fixed fresh.

With the exception of tissues to be examined histologically, fixation of host organs as a means of preserving parasites for later collection of parasites is not recommended. Fixed tissue hardens and it is virtually impossible to extract specimens from solid organs following fixation. If the intestine is preserved in this manner, it should be ligatured at each end (or in sections) and fixative injected into the lumen before it is placed in a larger container of fixative for storage. This will kill the parasites rapidly and minimize post mortem damage. Specimens killed in this way are fairly robust and cestodes are less likely to fragment than frozen ones. However, cestodes will often be strongly contracted making them difficult to study. Unless they were detached from the gut wall at the time of fixation, it is difficult to obtain intact cestodes or acanthocephalans. The rostellum of cestodes and the proboscis of acanthocephalans, are armed with hooks and are virtually impossible to dissect intact from fixed intestinal tissue. Another disadvantage is that gut contents may adhere to the surface of the specimens and will take up stain masking internal structures.

Ideally, all of the parasites from the host should be collected, cleaned and fixed as soon as possible after the host is killed. When this cannot be done, samples of parasites from the intestine of as many fresh hosts as is practical should be taken as quickly as possible and fixed for identification purposes. The remaining material should

be quick frozen or fixed and saved for counting. The tradeoff is that less common species may be missed in the taxonomic sample.

### *8.5. Examination Procedures - Examination of Organs*

The intestine and ceca should be examined first. They have the greatest diversity of parasites and the ones most likely to suffer post mortem damage in the short term. Parasites in other organs are affected less rapidly. Refrigeration of the organs for up to 24 hours has little effect on nematodes although the quality of digeneans will deteriorate. The sequence for examining the other organs is not critical but a good rule of thumb is to examine washes and hollow organs first, then the solid organs. Specimens found in each organ should be placed in water or saline in separate dishes until they can be processed.

#### *8.5.1. Intestinal Tract and Ceca*

Depending on the objectives of the study, the intestine may be divided into a few large sections or into a predetermined number of smaller sections for examination. Sections are opened by slitting them from anterior to posterior with scissors. Avoid a snipping action where possible; this may damage larger parasites. Parasites in the posterior half of the intestine are more robust so the duodenum and anterior regions should be examined first.

The ceca are a pair of blind, tubular, organs that join the large intestine slightly anterior to the cloaca. They are well developed in granivorous birds but are reduced and inconspicuous in carnivorous species. They should be examined immediately after the intestine.

1. Cover the opened section with water or saline and let it sit for a few moments while the parasites loosen and drop off. Most parasites will detach and die more quickly in water than in saline. Water is sufficient in most situations but in cases where cestodes fragment rapidly after death, saline is the better choice. Saline may also reduce mucous secretion by the gut of some host species and is worth trying if excess mucous is a problem. A weak solution of sodium bicarbonate (5%) may also reduce the amount of mucous in some instances.
2. Gently shake the gut section then pour the fluid and gut contents into a beaker. Cover the section with fresh water or saline and let it sit.
3. Let the contents of the beaker settle.
4. Decant the supernatant into another container and save it.
5. Examine the sediment. Larger specimens can be seen easily with the naked eye; 6X or 12X magnification is normally sufficient for smaller ones. Remove any large parasites and place them in a Petri dish of water or saline. Next, examine the sediment in small quantities (dilute if necessary) under magnification in a Petri dish. A Petri dish with grid lines scored on the underside is particularly useful for systematic examination of the material. Four careful passes through each dish of sediment are usually sufficient to determine whether parasites are present. These can be removed and saved as encountered. The sediment should be stirred between each pass. Additional passes will be necessary if there are large numbers of small parasites and should be repeated until all of the specimens have been removed.
6. Examine any mat of mucous floating on the surface of the supernatant fraction (Step #3); small helminths may be caught in it and not settle out. The mat can be drawn off easily with a pipette for examination as described in Step 5.
7. Examine any sediment that has settled in the supernatant fraction (Step #3).

8. Repeat Steps 1-7 until all of the gut contents have been examined and the mucosal surface is clean.
9. Finally, examine the mucosal surface with a dissecting microscope for any attached specimens and remove them (Section 8.5.1.1 below).

Alternatively, intestinal contents can be washed through sieves. Doster and Goater (1997) suggest using a No. 100 US standard sieve (opening size 0.15 mm) to screen helminths from gut contents, thereby reducing the amount of material to be searched (Step 5). This technique is best used with live, fresh material. Frozen specimens, particularly cestodes, may be too fragile to withstand this procedure.

#### *8.5.1.1. Removal of Attached Specimens*

Gentle suction, a stream of fluid from a pipette or careful scraping of the mucosal surface is usually sufficient to dislodge digeneans. Cestodes and acanthocephalans may need to be dissected free because the rostellum on the scolex of cestodes and the proboscis of acanthocephalans are frequently embedded in the intestinal wall. The hooks on these structures are important taxonomically and it is important to obtain them intact. Treat the cestodes carefully; rough handling may cause separation of the strobila and scolex resulting in the loss of the scolex and rostellar hooks. Acanthocephalans are more robust. The proboscis is usually embedded firmly in the intestinal wall and may be difficult to extract. The task can be facilitated by excising the attachment site from the intestine and leaving the specimen in water at room temperature for 24 hours. The tissue will decompose slightly allowing the proboscis to be dissected free more easily. This technique also works reasonably well for frozen material. It is virtually impossible to obtain an intact proboscis or rostellum from fixed tissue.

### *8.5.2. Examination of Body Cavity and Air Sac Washes*

Examine the washes from the body cavity and air sacs while those from the intestinal samples are settling. There is no mucous and little sediment in these washes so they can be examined quickly. Pour off the supernatant, and examine the sediment in a Petri dish under a dissection microscope. Intestinal parasites may be present in the washes if the bird was shot or if the intestine was damaged during removal.

### *8.5.3. Other Tubular Organs*

The trachea, esophagus, proventriculus, gizzard, gall bladder, ureters, bursa of Fabricius and oviduct are comparatively small and contain little or no solid material. Generally it is best to leave each organ intact until it is examined. Examine the external surfaces first, then open the organ and examine the inner surface and the contents.

#### *8.5.3.1. Esophagus*

Open the esophagus lengthwise, cover it with water and examine the external and internal surfaces using a dissecting microscope. A few species of digeneans and nematodes infect the lumen; other nematodes live underneath the mucosa and can be seen easily under low power magnification.

#### *8.5.3.2. Proventriculus*

Cut the proventriculus lengthwise and open it - do not add fluid at this point. Nematodes may occur in the yellowish mucous layer or in the proventricular glands beneath it. Digeneans have also been reported from the glands. Examine the mucous lining *in situ* with a dissecting microscope then scrape it off with a scalpel and examine it for nematodes. This is best done in a dry Petri dish. Tease and stretch the mucous with forceps or dissecting needles; the mucous will stick to the dry dish when stretched, facilitating examination. Once examination of mucous is completed, add water or saline

and examine the external surface and the lining. Nematodes belonging to *Tetrameres* and related genera are frequently present in the proventricular glands. These are usually large, dark red, and readily visible and will need to be dissected free of the tissue.

#### *8.5.3.3. Gizzard*

Gizzard worms are found primarily in waterbirds (Tuggle, 1987b; Anderson et al., 1996). Most are nematodes, but a specialized genus of cestodes (*Gastrotaenia)* also infects this organ. Open the gizzard and discard the contents, then, using forceps, peel off the koilin lining and place it in a dish of water. If the lining proves difficult to remove, soak the gizzard in water overnight. This will loosen the lining making it easier to remove (McLaughlin and McGurk, 1987). Most gizzard worms are either embedded in the lining or lie between it and the muscular surface. Examine the underside of the lining, the exposed gizzard surface and the wash water for parasites. Some nematodes of geese invade the muscle itself and it may be necessary to cut the gizzard into slices to find them (Tuggle, 1987b).

Other nematodes (*Echinuria* spp.) produce large granulomas located at the junction of the proventriculus and the gizzard. The worms are located in a cavity within the granuloma (that may or may not be open to the lumen) and can be recovered by dissection of it.

### *8.5.4. Trachea, Gallbladder, Bursa of Fabricius, Ureters and Oviduct*

Examine the external surface of the organ then open it, cover with water or saline and leave it for a few minutes. Shake the organ gently and allow the contents to settle. Pour off the fluid and examine the sediment for parasites. Two to three rinses of the organ are usually sufficient to dislodge all of the parasites present. Finally, examine the

inner surface with a dissecting microscope and remove any parasites adhering to it.

### *8.5.5. Circulatory System: Heart, Blood Vessels and Lymphatics*

Nematodes have been reported from the pericardium (normally observed before the heart is removed), heart musculature, near the valves of the heart and from the walls of major blood vessels (Wong et al., 1990). Parasites in the pericardium and in the heart musculature can often be seen with the naked eye but low power magnification is recommended and is essential for examination of the chambers and vessels. The major blood vessels can be examined by carefully opening the heart chambers and then dissecting the vessels leading from the atria. The walls of the pulmonary arteries and the lymphatic system may be infected with species of *Splendidofilaria* and *Chandlerella*, respectively (Bartlett and Anderson, 1985; Wong et al., 1990). In at least some instances, infected vessel walls are visibly thickened and enlarged (Huizinga et al., 1971).

Adult schistosomes are found in the venous system, especially the mesenteric and portal veins and veins of the nasal sinus of waterbirds. Younger worms may be present in the liver or the lungs. Specimens can be seen in the mesenteric veins with a dissecting microscope and can be removed by carefully dissecting them free with fine needles or forceps. Occasionally, specimens are present in the wash water from the body cavity or in the intestinal contents, having been freed from the mesenteries when they were detached from the intestine.

### *8.5.6. Examination of Solid Organs*

The liver, spleen, pancreas, and kidney are solid organs. Most also have ducts which should be opened under magnification with fine needles or forceps and examined. Examination of the rest of the organ requires a different approach. First,

examine the external surface of the organ with a dissecting microscope. If the organ is small it can be teased apart in water or saline under magnification and searched for parasites. Cut larger organs into smaller pieces and press each piece between two glass plates. The preparation can then be examined with a dissecting microscope for parasites. Parasites from these sites seem particularly susceptible to osmotic shock and should be placed in saline rather than water until they can be fixed. This technique works best with fresh material. Except for nematodes, it does not work well for frozen material and is of no use for organs that have been fixed.

### *8.5.7. Lungs*

Examine the external surfaces for parasites or lesions. Lung tissue (parenchyma and air passages) can be examined by dissection under magnification using dissection needles and fine forceps or scissors. It can also be cut into small pieces and examined in a press mount between two plates of glass as described for solid organs, although trapped air will make observation difficult. This can be overcome to a large extent placing the tissue under vacuum before examining it.

### *8.5.8. Musculature, Skin and Joints*

*Sarcocystis* (Phylum Apicomplexa) occurs in the breast and other skeletal muscles. Although a protozoan, the cysts of this parasite are large enough to be seen with the unaided eye and appear as white rice grain-sized bodies in the tissue. *Sarcocystis* may also infect the heart (Tuggle, 1987a). Any other suspect lesions should be investigated. Excise some of the material and tease it apart under low power magnification and examine as a temporary mount with a compound microscope.

Nematodes (mostly filarial and dracunculoid species) occur in subcutaneous sites (Wobeser, 1981, 1997; Wong et al., 1990). Different species occur in the

connective tissue around the esophagus and trachea in the neck, in subcutaneous sites and in ankle and knee joints (Wong et al., 1990). Most are large and easily detected in the tissue. Digeneans belonging to the genus *Collyriculum* also occur in birds in subcutaneous cysts that open to the outside. (Byrd, 1970; Denzler and Lopsiger Molliet, 1991).

### *8.5.9. Examination of the Head*

Most of the parasites in the head infect the sinuses. To examine the sinuses, cut the bill transversely through the nares then extend the cut backwards on either of the head to a point just below the eyes. Bend the upper part of the bill backwards to expose the sinuses. Digeneans and nematodes are readily visible and can be removed easily. Leeches and mites, covered in Section 9, may also be present. Nasal schistosomes may be visible when the sinuses are viewed under magnification (see Section 8.5.5 above).

Few parasites occur in the buccal cavity although *Clinostomum* (Digenea) is common in herons and nematodes have been reported under the tongue of birds. The louse *Paigetiella* is a frequent parasite of the throat pouches of pelicans and cormorants (Wobeser et al., 1974).

Digeneans and nematodes may be found under the nictitating membrane of the eye and in the conjunctival sacs (McDonald, 1974, 1981). They can be rinsed free using a gentle stream of water or dissected free of the surrounding tissues.

Few parasites infect the brain. If the brain is to be examined, cut the skin on the head to expose the skull. Carefully remove the skull cap to expose the brain and gently lift it out. Examine the surface with a dissecting microscope for nematodes. Filarial

nematodes have been reported from the pia mater (Bartlett and Anderson, 1980). Birds displaying neurological symptoms should be examined for cerebrospinal nematode infections. At least two cases of cerebrospinal nematodiasis due to *Baylisascaris* are known from birds (Armstrong et al., 1989; Evans and Tangredi, 1989).

### 8.6. SAMPLE COLLECTION - MICROPARASITES

There are number of protozoan parasites that infect birds and a good account of these can be found in Doster and Goater (1997). They also discuss a number of diagnostic techniques for detecting protozoans including several (serology, isodiagnosis culturing and tissue smears) that are beyond the scope of this review. Only the coccidians and flagellates will be considered here.

Coccidians (Phylum Apicomplexa) are intracellular parasites. They are typically host and site specific. Most species infect the intestinal mucosa but some species infect other organs, particularly the kidneys. Coccidian infections produce inflammation and lesions in the mucosa and swelling with mottling and white foci in kidneys (Wobeser, 1981). Infections can be detected by microscopic examination of cells obtained by scraping lesions and in histological sections of the lesions (Wobeser 1981, 1997). Reid et al. (1983) and McDougald (1983) provide excellent descriptions of some of the common species of coccidians and other protozoans in poultry and the lesions caused by them. Lesions present in wild species would be similar.

Identification of coccidian species is based on the morphology of the sporulated oocyst (the transmission stage) (Wobeser, 1981, 1997). These must be isolated from the feces and cultured until they sporulate. Unless they are the object of the study, oocysts are not collected in survey work. Todd and Hammond (1971) describe the oocysts of several common species found in wild birds and methods for collecting them.

*Trichomonas gallinae* occurs in the crops of pigeons and occasionally other birds that either share feeding or watering sites with them or prey upon them. Other species have been reported from the ceca. Trichomonads can be detected in fresh smears of the crop or cecal contents (Doster and Goater, 1997).

## 8.7. SAMPLE PREPARATION - MACROPARASITES - Fresh Material

### *8.7.1. Relaxation and Washing Specimens*

Live helminth specimens, particularly cestodes and digeneans, are usually allowed to "relax" or extend in water before being fixed. This is the result of the animal becoming moribund and extended due to osmotic phenomena. Smaller specimens "relax" more quickly than larger ones and some authors suggest placing larger cestodes in a refrigerator until they are fully extended. The process has no effect on parasites that are already dead.

Specimens should be cleaned of all debris and mucous before being fixed. Small specimens can be cleaned by gently squirting them in and out of a Pasteur pipette in a small dish of clean water. Digeneans, nematodes and acanthocephalans can also be cleaned by placing them in a small vial half full of water and shaking vigorously for a few seconds. Allow the parasites to settle, pour off the supernatant and repeat if necessary. Examine the supernatant after it settles for any specimens that may have been lost. DO NOT use this procedure for cestodes; they will fragment or tangle. Small cestodes should be cleaned gently by pipette as described above. Larger more robust specimens can be grasped with light forceps or supported by a dissecting needle and gently agitated in the wash water to remove any material from the surface. It is particularly important to ensure that the rostellum (cestodes) and the proboscis (acanthocephalans) are free of debris so the hooks can be examined properly.

### 8.7.2. Fixation and Storage: Macroparasites

Fixation is the rapid killing of the specimen in a manner that minimizes distortion. Commonly used fixatives include 5% -10% buffered formalin, acetic acid-formalin-alcohol (AFA or FAA), Bouin's fluid and hot 70% ethanol. Recipes for two frequently used fixatives, acetic acid-formalin-alcohol (AFA or FAA) and Bouin's fluid, are given in Appendix V. Recipes for other fixatives can be found in Humason (1972) and similar sources. Specimens are usually left in the fixative for 24 hours but may be stored in some fixatives indefinitely.

Following fixation, specimens and tissues should be stored in 70% ethanol in tightly capped vials or other suitable containers until they can be studied. Specimens from each organ should be kept in separate containers. If possible, nematodes should be kept separately from other helminths because they are usually stored in a different fluid (see Section 8.7.2.3). A label written in pencil or indelible ink, containing information on the host (see Sections 3.1 and 3.2, in part) should be placed inside each container. Labels on the outside get lost easily.

#### *8.7.2.1. Digeneans*

Following washing, small specimens can be placed directly into fixative. Large specimens or species that tend to curl may require flattening under light pressure from a cover glass during fixation to keep them straight. Avoid excessive flattening. This will distort internal organs (and their measurements). Bakke (1988) evaluated the suitability of 22 fixation and processing techniques on digeneans. Hot fixatives ( 4% or 10% formalin-saline, 10% buffered formalin, AFA and 70% ethanol) and Berland's fluid at room temperature gave the best results. Store specimens in 70% ethanol.

#### *8.7.2.2. Cestodes*

Following cleaning and relaxation, small cestodes can be placed directly into fixative. Five to 10% buffered formalin and AFA are commonly used fixatives and are more effective when used hot. Hot 70% ethanol is also effective. Larger specimens should be grasped by the posterior end and swirled through a dish of fixative until they die to minimize contraction. Long cestodes should be left in a large dish of fixative until they have hardened slightly before being placed in vials. Store specimens in 70% ethanol.

#### *8.7.2.3. Nematodes*

Living nematodes are fixed in hot 70% ethanol. The hot fixative causes them to straighten out making them easier to study. This procedure has no effect on dead specimens. Nematodes are usually stored in 5% glycerine in 70% ethanol following fixation rather than 70% ethanol.

#### *8.7.2.4. Acanthocephalans*

Following cleaning (with particular attention to the proboscis) specimens can be placed directly in fixative (5%-10% buffered formalin, AFA or hot 70% ethanol). Store specimens in 70% ethanol.

### 8.8. SAMPLE PREPARATION - MICROPARASITES

Infected tissue can be fixed in 5-10% buffered formalin, AFA, Bouin's or other suitable fixative for 24 hours or longer. Smears can be fixed in Schaudinn’s or similar fixative (Appendix V). Tissue samples can be stored in some fixatives indefinitely, however, the usual practise is to store specimens in 70% ethanol after fixation. Smears are normally processed immediately after they are fixed.

## 8.9. EXAMINATION RECORDS

Records of the organs infected, the type of parasites found in each organ and the fixative used are useful for keeping track of material collected from a particular host. It is also useful to include a list of the vials containing specimens and a list of material that has been frozen or fixed in bulk. This will also help organize the taxonomic and quantitative aspects of the work. See Appendix VI.

## 8.10. STAINING

With the exception of nematodes which are normally examined unstained, helminths and smears or sections containing protozoans are stained prior to examination. The fixative will interfere with the staining process and must be removed by placing the specimens in 70% ethanol for 24 to 48 hours prior to staining. Helminths are stained routinely in acetocarmine which is commercially available and is easy to use (See Appendix VIII). A number of other stains such as Schneider's carmine and Ehrlich's, Delafield's and Van Cleave's haematoxylins also produce excellent results but the procedures are slightly more complicated.

Following staining, the specimens are dehydrated and cleared following standard histological procedures, mounted individually on microscope slides in Canada balsam, or in a synthetic mounting medium such as Permount, and studied as whole mounts. An overview of staining procedures is presented in Appendix VII. Specific instructions for routine staining of specimens for whole mounts and for staining sections and smears are presented in Appendix VIII and IX, respectively.

Each slide should be permanently identified with a code number, unique to the particular host. Once identified, a label bearing name of the parasite and the host, the site within the host, the code number for the host and the collection date and locality should be applied.

### *8.10.1. Digeneans*

Digeneans are studied as whole mounts. Acteocarmine (see Appendix VIII) and Schneider's carmine give excellent results.

### *8.10.2. Cestodes*

Cestodes are studied as whole mounts. Routine staining can be done in acetocarmine (see Appendix VIII) but the haematoxylin stains also produce excellent results. **Special technique:** to facilitate the observation, counting and measurement of the rostellar hooks, cut off the tip of the rostellum, mount it on a microscope slide in Hoyer's or Berlese Fluid and add a cover glass. When the preparation is dry, ring the cover glass with a waterproof sealant. Because the tissue clears quickly and only the hooks remain visible, it is advisable to draw a ring around the rostellum before it clears to make them easier to locate.

### *8.10.3. Nematodes*

After fixing, nematodes are usually stored in a solution of 5% glycerine in 70% ethanol. Normally, nematodes are not stained. They are studied as temporary mounts after being cleared in glycerol or lactophenol. Ash and Orihel (1991) suggest transferring the specimens through a series of increasing concentrations of glycerol in 70% ethanol (a process similar to the dehydration process described in Appendix VIII) until they are in pure glycerol, at which point they are clear and ready for study. An alternative method is to place the specimens in a solution of 5% glycerol in 70% ethanol in a loosely covered container. The ethanol is allowed to evaporate slowly over several days leaving the cleared specimens in a thin film of glycerol. The specimens are studied as temporary mounts in glycerine.

Nematodes can also be cleared in lactophenol. Specimens placed directly in lactophenol from alcohol will clear quickly. The lactophenol can be removed in 70% alcohol after examination if necessary.

Semipermanent mounts of nematodes can be made in glycerine jelly, which is commercially available. Specimens cleared in glycerol are transferred to a drop of molten jelly on a microscope slide and a cover glass is added. After the jelly has hardened, ring the edge of the cover glass with a sealant. If necessary, the jelly can be melted to reposition the specimen or to return it to the vial for storage. Permanent mounts of stained or unstained nematodes can made in Canada balsam or in some other mounting medium after dehydration and clearing as described AppendixVIII. **Special Technique:** Identification of nematodes frequently requires observation of cephalic structures. These are best seen in *en face* views. The anterior tip of a worm is severed and mounted, face down, in a tiny drop of molten glycerine jelly on a cover glass (Anderson, 1958). A drop of glycerine jelly is placed on each corner of the cover glass. The cover glass is then inverted and placed on a standard microscope slide for viewing.

### *8.10.4. Acanthocephalans*

Acanthocephalans are studied as whole mounts and can be stained in acetocarmine (see Appendix VIII). **Special technique:** the number of rows of proboscis hooks is an important taxonomic feature. Where it is difficult to count the rows accurately, the proboscis can be cut off and mounted *en face* in glycerine jelly as described above for nematodes (see Section 8.10.3 above) to facilitate the task.

## 8.11. SAMPLE PREPARATION - MICROPARASITES

Tissue specimens containing suspected parasites are embedded in paraffin and sectioned at 8-10□m. The sections are mounted on microscope slides and stained in Ehrlich's or Delafield's haematoxylin and counterstained in eosin following standard histological procedures. The procedure for routine staining of sections with Ehrlich's haematoxylin and eosin is described in Appendix IX. A modified version of this procedure (see Appendix IX) is used to stain smears.

## 8.12. SAMPLE PREPARATION - Material from Frozen or Fixed Organs / Tissues

The procedures for examining frozen or fixed host material for macroparasites are the same as those described above except that it is pointless to make press mounts of fixed tissues. Generally, frozen specimens tend to be more fragile and cestodes, in particular, tend to fragment easily. Specimens should be cleaned as well as possible; however, as they are already dead, the fixation steps are unnecessary and they should be placed directly into 70% ethanol, regardless of taxon. Specimens can stained as described above but as a general rule will not stain as well as those fixed fresh. Freezing usually destroys microparasites.

## 8.13. IDENTIFICATION

Microparasites and macroparasites are identified to the generic level on morphological criteria and to species level on a combination of morphological and morphometric criteria. Those data have traditionally been obtained with a microscope and an ocular micrometer. Digitizing equipment is now available that will do the same job. Regardless, some sort of descriptive data sheet or form, listing the various observations or measurements to be taken, is needed. Requirements vary from group to group so the data sheets are usually custom made for the particular group under

consideration. See Appendix X for an example of a generalized data sheet for a digenean. The data sheets contain the raw measurements on each specimen in ocular units as well as morphological notations and contain information that is unlikely to be included in databases or spreadsheets. Record the magnification for each measurement because the conversion factor differs at each magnification.

Keep a record of the slides made from each host and record the name of each specimen as it is identified. Reference slides and potential voucher slides can be identified and set aside as the work progresses. When all of the species from the host have been identified, the rest of the specimens from the host can be sorted and counted.

References to keys for the identification of protozoan and helminth parasites are listed in Appendix XII.

## 8.14. QUANTIFICATION

### *8.14.1. Macroparasites*

After the parasites in a host have been identified, the next step is to determine the number of each species present. Doster and Goater (1997) provide a thorough review of procedures and problems associated quantification of endoparasites in individual hosts. Generally speaking, is usually easier to get accurate counts from hollow organs than from solid ones. With the exception of the intestine, diversity is low, few individuals will be present and there is only a small volume of material to examine. Quantification of intestinal parasites can be more difficult, particularly in larger hosts, owing to the larger populations, greater diversity and the volume of material that must be examined. Larger parasites can be counted accurately but it may be difficult to get accurate counts of smaller species. Fresh material is easier to work with than frozen or

fixed material however, digeneans, nematodes and acanthocephalans are robust and remain intact regardless of preservation technique. Unfortunately, cestodes often fragment when handled following freezing and many species fragment even when fresh, making it difficult to obtain accurate counts. Small specimens of all taxa may get trapped within clumps of intestinal debris if the intestinal contents have been fixed and may be missed.

Most of the organs will only contain a few parasites and the normal procedure is to mount and identify all of them and determine the number of each species directly. Where moderate or large numbers are present, as is often the case with the intestine, it is not practical to stain and identify all of the specimens. Instead, a sample of specimens selected on the basis of general appearance is stained and identified. Once the number of species has been determined, the remaining specimens are sorted and counted. Where large numbers are present, a sample of the specimens is selected (from the taxomonic sample if one was taken) and identified. Once the species have been identified, the number of each species can be determined using subsampling procedures described in Section 8.14.2.

**Special technique:** Sorting specimens for counting is straightforward when the species can be easily distinguished. However, birds are often infected with a number of species (particularly cestodes) that are difficult to distinguish from each other with a dissecting microscope. A quick way to sort cestodes that cannot be distinguished easily is to mount them in either Berlese's fluid or Hoyer's medium. If the cestodes are small, the entire worm can be mounted; a few proglottids from the mature section of larger specimens is usually sufficient. The  tissue clears quickly but the rostellar hooks (if present) and details of the cirrus sac and cirrus remain visible and can be readily observed with a compound microscope. These can then be compared with stained

specimens for identification. Unfortunately, such specimens are not suitable for archival material nor can they be retrieved for more critical study. This method also works well with cestodes from frozen or fixed samples which typically stain poorly, if at all.

### *8.14.2. Subsampling*

When large numbers of small helminths are present (often in the hundreds or thousands), estimates of the total load are made by subsampling. Remove all of the large parasites and count them, then dilute the remaining material to some fixed volume (e.g. 100 ml). Mix gently, but thoroughly, and take three subsamples. The specimens in each subsample are identified and counted. If the number of each species from the subsamples is similar (e.g. differs by less than 10% [Edwards and Bush, 1989]) the samples can be considered homogenous. If not, additional subsamples should be taken to get a better estimate. The number of each species in the total sample can be estimated by summing the counts for each subsample and adjusting the counts to the final volume (Edwards and Bush, 1989) or by averaging the counts over the subsamples and multiplying by the total volume (Alexander and McLaughlin, 1997).

### *8.14.3 Microparasites*

Quantification of microparasites is not usually done in examinations of this type. However, the location of lesions, the number of lesions present and their size may be of interest depending on the study.

# 9. ECTOPARASITES

## 9.1. INTRODUCTION

Ectoparasites, for purposes of this discussion, include the leeches and a variety of arthropods such as lice, fleas, ticks and mites. These are listed along with their anatomical sites in Appendix I. A more detailed listing can be found in Clayton and

Walther (1997). The majority of ectoparasites are associated with the skin and feathers but some species infect subcutaneous sites (mites, flies) or internal sites such as the respiratory system (leeches and mites) and oral pouches (lice). Leeches are included here for convenience although some might argue that they are not strictly ectoparasites. Some ectoparasites are permanent and spend their life on the host. Others spend part of their life on the host and some are only associated with a host for brief periods at a time. Clayton and Walther (1997) provide an excellent overview of arthropod parasites of birds and techniques for their collection, preservation and storage. They also provide references to a number of useful reviews on ticks, lice, mites and parasitic insects. The following section is based largely on their review and on information provided by Furman and Catts (1982).

## 9.2. COLLECTION

Permanent and temporary ectoparasites can be collected from either living or freshly killed hosts. Permanent parasites can also be obtained from preserved hosts (Clayton and Walther, 1997). Because some ectoparasites can leave the host, each individual should be placed in a separate container immediately after capture or death to ensure that no parasites are lost and that none transfer for one host to another. While several options exist, Clayton and Walther (1997) suggest the use of heavy paper lunch bags to isolate small live birds. Larger live birds require larger and stronger containers. Paper or plastic bags are suitable for dead birds. In all cases, the containers must also be examined as parasites often leave the host after death. Many parasites are visible with the naked eye but a dissecting microscope or some other magnifier (Clayton and Walther suggest a jeweller's head set) is required for smaller species.

### *9.2.1. Collection of Ectoparasites from Live Hosts*

Ectoparasites can be removed manually from living hosts, however quantification of the parasite loads may be problematic. The technique is accurate for some of the larger stationary parasites like leeches, ticks and some lice (e.g. *Piagetiella* in the pouches of pelicans) which can be located with the unaided eye, but it is less so for smaller or more mobile ones. With appropriate magnification, smaller parasites like feather mites, can be located on the flight and tail feathers. These can be removed by dislodging them with a dissecting needle or some similar instrument. Skin and scale mites can be collected from scrapings of the microscopic lesions they produce. On the other hand, collection of quill lice and quill mites requires dissection of the actual shafts of flight feathers and is best done with the aid of a dissecting microscope.

Alternative approaches involve the use of anaesthetics and insecticidal powders to kill the parasites *in situ* after which the feathers are ruffled to dislodge them. In the anaesthetic procedure, a bird is placed in large jar with a special lid that permits the head to protrude. Chloroform, ether or some other volatile anaesthetic is added to filter paper in the bottom of the jar. The bird is restrained with its body in the jar for 20 minutes or so (Clayton and Walther, 1997). During this time many of the dying parasites detach and fall off. On removal, the bird is held over the jar or some other collecting surface and the feathers are ruffled to dislodge additional parasites.

More recently, insecticidal powders have replaced the chemicals traditionally used in this procedure. First, the bird is dusted with the powder and is then placed in a confined area over a collecting surface for a fixed period. During this period, most of the parasites die and fall off. The feathers are then ruffled while the bird is held over the collecting surface to recover the reminder. This method is effective for a greater variety of parasites and removes a greater number of parasites overall than the anaesthetic

technique. It also permits collection of parasites from the head which is not possible with the anaesthetic technique and eliminates the dangers inherent with the use of some anaesthetics (Clayton and Walther, 1997).

### *9.2.2. Collection of Ectoparasites from Dead Hosts*

Hosts should be placed in individual bags immediately after death to prevent the loss of temporary parasites and contamination of other hosts. Although best results are obtained from freshly killed hosts, specimens can be obtained from refrigerated, deep frozen, preserved or embalmed birds (Clayton and Walther, 1997). Visual examination of the carcass and the anaesthetic and dusting techniques described above for live hosts can also be used to collect arthropods from recently killed hosts. Visual examination and feather ruffling techniques may also be used when the parasites have been killed by freezing or other forms of preservation.

More destructive examination methods include the systematic removal and examination of individual feathers, washing of the carcass and dissolving of the feathers and skin. To wash the carcass, place it in a dilute detergent solution and agitate vigorously for 5-10 minutes. Remove the carcass, allow the fluid to settle then pour off the supernatant and examine the sediment for parasites. The supernatant should also be allowed settle and any additional sediment examined to ensure that no specimens are lost. Dissolution techniques involve skinning the specimen and dissolving the skin and feathers. These are incubated in an enzyme bath (trypsin) at an appropriate pH for 24 hours then boiled in KOH until the skin and feathers are dissolved. The fluid is then passed through a screen to collect the parasites (which actually are only exoskeletons at this point). These are washed off the screen and allowed to settle for further processing. See Clayton and Walther (1977) for further details. Some authors report that flotation techniques, similar to those used to separate benthic invertebrates from

sediments, are helpful in collection parasites following washing or digestion procedures (Clayton and Walther, 1997).

### *9.2.3. Examination of Internal Sites*

Parasites in internal sites are usually collected at necropsy. Leeches are normally restricted to the nasal sinuses; mites, and occasionally other arthropods, can be found throughout the respiratory system and body cavity. Mites may also occur in the ears. Procedures for exposing and removing the organs are described in Section 8.2 and 8.3, respectively. Examination procedures for specific organs appropriate to this section can be found in Section 8.5. See subsections 8.5.2 (body cavity and air sacs), 8.5.4 (trachea), 8.5.7 (lungs), and 8.5.9 (oral cavity and sinuses) for details. Leeches are large enough to see with the unaided eye. Mites require magnification and can be viewed in *situ* (usually with some difficulty) and in the sediment of rinse water from these sites.

### *9.2.4. Myiasis*

Myiasis is an infestation by fly larvae of living, necrotic or dead vertebrate tissue (James, 1982). Larvae of myisais-causing flies occupy conspicuous, swollen chambers just under the skin that communicate to the exterior by a pore. They eventually exit from the host through the pore and drop to the ground where they pupate. The larvae can be removed through the pore with forceps from live or dead hosts and by dissection if the host is dead.

## 9. 3. SAMPLE PREPARATION

### 9.3.1. Relaxation, Fixation and Storage

#### *9.3.1.1 Hirudinea*

Specimens must be narcotized prior to fixation otherwise they will contract, making them difficult to identify. Davies (1991) recommends gradual addition of 95% methanol to water containing leeches, $CO_2$ bubbled through water or Nembutal as narcotizing agents. Specimens can be fixed in 70% ethanol (recommended) or in 5% buffered formalin if colour preservation is an issue. Gentle flattening of the specimen between two microscope slides during fixation may be necessary. Davies (1991) recommends injection of larger specimens with fixative once they are dead for better preservation, although this is rarely necessary with the species found on birds. Store specimens in 70% ethanol or 5% formalin as required in tightly capped vials or other containers. Include labels providing information on the host and site where the specimens were found (see Section 8.7.2 for details).

#### *9.3.1.2. Arthropoda*

Arthropods are normally fixed in 70% ethanol or in isopropyl alcohol (Furman and Catts, 1982). Specimens are normally stored in 70% ethanol in tightly capped vials. Include appropriate labels (see Section 8.7.2 for details).

## 9.4. STAINING AND MOUNTING

### *9.4.1. Hirudinea*

Specimens can be stained in carmine or haematoxylin stains and prepared as whole mounts as outlined in Section 8.10 and Appendix VIII. Additional procedures are described in Davies (1991).

### *9.4.2. Arthropods*

Specimens are not stained; rather, they are mounted directly from water or alcohol on microscope slides in a drop of Hoyer's medium or Berlese fluid and covered with a cover glass. These media clear the soft tissues quickly but leave the cuticular structures intact. The edge of the cover glass should be ringed with a sealant once the preparation is dry. Specimens can also be mounted permanently on slides with Canada balsam, or a synthetic mounting medium, following the standard dehydration and clearing steps outlined in Appendix VIII.

## 9. 5. IDENTIFICATION

References to keys for the identification of leeches and arthropods are listed in Appendix XIII.

## 9.6. QUANTIFICATION

Leeches and large arthropods, like ticks and pouch lice, can be counted directly and there is little problem obtaining accurate counts from each host. Smaller species and species that live under the skin are far more difficult to enumerate. Clayton and Walther (1997) provide a thorough discussion of the difficulties associated with the quantification of arthropod parasites and evaluate various techniques used for particular parasites. Overall results vary depending on the type of parasite (permanent vs temporary) being studied, whether the host was alive or dead, and the collection techniques used. Generally, dusting and washing techniques produce the greatest numbers of parasites from live and dead hosts, respectively, and more accurate counts can be made from dead hosts than from live ones (Clayton and Walther, 1997). This review should be read prior to any study requiring the quantification of ectoparasites.

# 10. BLOOD PARASITES

## 10.1. INTRODUCTION

A variety of blood protozoans including *Trypanosoma*, *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* (Bennett et al., 1989) infect birds. The latter three genera infect erythrocytes (*Leucocytozoon* also infects white blood cells) while *Trypanosoma* and the microfilariae of a number of filarial nematodes (Wong et al., 1990) occur in the plasma. Blood samples can be obtained easily from live hosts and large numbers of birds can be sampled during banding surveys. There is an extensive literature on avian haematozoans.

## 10.2. COLLECTION

Blood samples are normally taken from live birds, usually from a vein in the wing or leg. Blood smears may be made from clots or blood in the chambers of the heart shortly after death and from frozen specimens but the results are generally poor (Bennett, 1970). Thin blood smears are essential due to the nucleated condition of avian red blood cells (Bennett, 1970). To make a thin smear, place a drop of blood at one end of an alcohol-cleaned microscope slide. A second slide, applied at a $45^{o}$ angle to the first, is touched to the drop of blood which flows along the edge. The slide is then drawn or pushed along the first towards the opposite end, producing a film of blood that becomes thinner with increasing distance from the original drop. See Bennett (1970) and Doster and Goater (1997) for further details. Smears are air dried and should be fixed as soon as possible in 100% ethanol or 100% methanol (Bennett, 1970) and stored in a cool, dust free environment until they are stained and examined. Godfrey et al. (1987) suggest more than one smear per host be made. Slides should be clearly identified, by pencil if they have frosted ends or with a diamond or carbide marker if they do not. All pertinent collection data should be recorded at the time.

## 10.3. SAMPLE PREPARATION

Blood smears must be stained prior to study. Giemsa's stain is one of the standard stains used but others are available. Smears must be fixed before staining in Giemsa. The staining procedure is outlined in Appendix XI. Other procedures can be found in Garcia and Ash (1979) and Ash and Orihel (1991). A variety of stains are available commercially in concentrated form. Follow manufacturers protocols when using other stains.

After the slide has dried, apply a #1 cover glass directly to the smear with Canada balsam or some synthetic mounting medium. Once the mounting medium has dried the slide can be studied. Trypanosomes and microfilariae will be interspersed among the blood cells, *Plasmodium Haemoproteus* and *Leucocytozoon*, will be found in erythrocytes; *Leucocytozoon* will also be found in leucocytes.

## 10. 4. IDENTIFICATION

A list of references providing keys for the identification of hematozoan species is given
in Appendix XIV.

## 10.5. QUANTIFICATION

Infections are detected by microscopic examination of smears, normally at 1000X for protozoans and at lower magnifications for microfilariae. Traditionally, a fixed number of fields is examined per smear or the smear is examined for a fixed period of time (or some combination of the two approaches is used) before a smear is considered negative. Usually only prevalence (percentage of hosts infected) is reported.

Seegar (1979) evaluated four methods for detecting microfilariae in swan blood.

These included thin smears, Knott's test, wet mounts and serum centrifuged in capillary tubes. The latter two were the most sensitive for detecting microfilariae; thin smears were the least effective. Filarial worm infections are typically underestimated because microfilariae are not produced in single sex infections.

Accurate estimates of intensity (number of a parasites in a host individual) of blood parasites are difficult to obtain. Godfrey et al. (1987) discuss the problems associated with attempts to quantify haematozoans, the need for standardized procedures and provide a number of recommendations toward this end. For intraerythrocytic parasites, intensity of infection is normally based on estimates derived from examination of 10,000 to 20,000 erythrocytes and is expressed as the mean number of parasites / n erythrocytes (e.g. mean ± SE / 10,000 erythrocytes obtained from 100 cells from 100 replicate fields [Fedynich et al., 1993]).

Techniques for quantifying intracellular haematozoans that infect erythrocytes and leucocytes and microfilariae are not available (Fedynich et al., 1993).

## 11. VOUCHER SPECIMENS

Type specimens of any new taxon described must be deposited in a museum or in a recognized collection. Many journals now require that voucher specimens of species reported in surveys be deposited as well. The accession number of each specimen should appear in the publication. Huber (1998) provides an excellent discussion of the importance of voucher specimens and the role of taxonomists and non-taxonomists in establishing and maintaining them. Voucher specimens are the only way an identification can be verified. They are particularly important when pests, potential pathogens or species previously unrecorded from a particular region, country or continent are reported.

Voucher specimens submitted for permanent reference should be intact, well prepared, mature individuals. Several specimens of each species and, if appropriate, several specimens of each sex should be submitted if at all possible (Huber, 1998). Specimens submitted on slides should be mounted in Canada balsam; synthetic media are generally not suitable for archival material.

Appropriate documentation including the collection locality (country, province, county or parish, local site), date (spell out the month or use Roman numerals), host species, parasite species if known (scientific names), site within the host, the fixation and preservation technique used and the name of the collector should be included (list modified from Huber, 1998). If available, the name of the person making the identification and the publication where the specimen is referred to should also be included.

Avian haematozoans should be submitted to the Center for Avian Haematozoa, Queensland Museum, Australia. In Canada, helminth specimens can be submitted to the Canadian Museum of Nature, Ottawa. Lichtenfels and Ward (2000) list addresses for the major parasite collections in the United States (helminths, leeches, crustaceans, ticks, mites, and insects). Other major institutions e.g. the British Museum (Natural History), London, will accept specimens. Contact the curator for instructions before shipment.

Frey et al. (1992) have suggested that a host specimen (symbiotype) should also be submitted when describing new parasite species. The authors point out that important collateral information can be obtained from such specimens. They offer several suggestions regarding the submission and curation of these specimens.

# D. DATA ANALYSIS

## 1. VERIFICATION PROCEDURES

Identifications of host and parasite species should be confirmed by an expert, if possible. This is especially important if there is any question regarding the accuracy of a particular identification. Data entered into spreadsheets or databases should be proof read carefully. This is best done by two people and is essential before any analyses are done. Make copies of all original log books, data sheets and computer files and store them separately.

## 2. DATABASE MANAGEMENT PROCEDURES

Ideally one, or at most, a few trained individuals should enter the data and maintain the database. Those responsible should maintain and update files and ensure that software is upgraded as appropriate so that the databases remain accessible over time. Databases should be backed up routinely as new data are added. Electronic and hard copies should be stored in a safe location.

## 3. DATA INTERPRETATION

Each host collected from the same habitat during the same time frame is considered a replicate of the others (Holmes and Price, 1986). Thus, the parasite populations or the parasite community within each host are also replicates. Three common measures used to describe infection levels in a sample are prevalence, abundance and intensity. Bush et al. (1997) offer a number of observations and suggestions regarding the use of these descriptors. Prevalence is the proportion of hosts in a sample infected by a particular parasite and is usually expressed as a percentage. While sample size has no effect on maximum prevalence (it is always 100%) it does influence minimum prevalence values. If only one individual in sample of 5, 10, 50 and 100 hosts is infected the minimum prevalence for each is 20%, 10%, 2%

and 1%, respectively (Gregory and Blackburn, 1991). Inclusion of the sample size is essential for comparative purposes. Comparison of prevalences between samples is normally done using Chi-square tests or, preferably, with Fisher's exact test (Rósza et al., 2000).

Mean abundance (the mean number of a particular parasite species per host in the sample) and mean intensity (the mean number of a particular parasite species per infected host in the sample) are the most commonly used quantitative measures. Generally parasite populations have aggregated distributions and arithmetic means can be distorted badly by a few extreme values. Rósza et al. (2000) discuss the advantages and drawbacks of using means, medians and geometric means to describe abundance and intensity. They state that frequency distributions are among the most informative ways to quantify parasites in a sample and suggest that confidence intervals, rather than standard deviations, be given. They suggest bootstrap methods for calculating confidence intervals when the number of infected individuals is too small (30) to be constructed by usual methods. They include appendices describing updated computations of confidence intervals and intensities.

If the data can be normalized using transformations they can be analysed using parametric statistics, otherwise nonparametric statistics have traditionally been used. Rósza et al. (2000) suggest additional tests (e.g. randomization tests) for comparing distributions of parasites among samples.

The population of a parasite within a single host is referred to as an infrapopulation, that within a host population as the component population and that within all hosts of a given species within the ecosystem as the suprapopulation. Parallel concepts exist at the community level and include the infracommunity which includes all

of the parasites in an individual host, the component community which includes the infracommunities of all hosts within the host population (but see Bush et al., 1997 p. 582 for a discussion of this concept), and supracommunity which encompasses all suprapopulations (Bush et al., 1997).

A variety of diversity indices have been used in the study of parasite communities. Species richness, mean percent similarity, Brillouin's Index, Shannon-Wiener Index and various eveness indices have been used by various authors. A variety of multivariate procedures, including principal components analysis, cluster analysis and multidimensional scaling, have been used to analyse helminth communities within the same species from different locations (Bush and Holmes, 1986; Levy, 1997) or in sympatric host species (Stock and Holmes, 1987; Alexander and McLaughlin, 1997).

## E. QUALITY ASSESSMENT AND QUALITY CONTROL

Use of standardized data sheets at various stages in the process (e.g. collection, necropsy, identification, sorting and enumeration) will ensure that comparable information is collected from each host in the sample. In addition, constant monitoring at each step is essential. A rigorous monitoring protocol should be designed as an integral part of any study and sufficient resources devoted to it. Identifications, particularly those establishing new host or geographic records, should be verified by an expert.

## F. VOLUNTEER (NON-SPECIALIST) INVOLVEMENT

Because of permit requirements, casual volunteers cannot normally assist in the collection of hosts. Specimens may be obtained from hunters who can contribute legally killed hosts for study, and large samples of waterfowl tracheae and gizzards have been obtained in this way (Scott et al., 1979; McLaughlin and McGurk, 1987). Specimens can

also be obtained in conjunction with other scientific activities, e.g. blood samples from banding studies or carcasses from capture mortality in banding operations. Volunteers and the general public can be an important source of information on injured, sick or dead birds. Landowners make important contributions by permitting collecting activity on their properties.

Examination of hosts for parasites is labourious and it is preferable to have a few experienced individuals work on this aspect of a study. However, with the exception of the intestine, most organs are relatively easy to examine and could be entrusted to appropriately trained volunteers. Similarly, data entry and basic staining and mounting techniques can be easily mastered by volunteer assistants. In situations where parasite diversity is low or where species are easily distinguished, volunteers could be helpful in sorting and counting specimens.

## G. SUMMARY

Parasites are part of the overall diversity of ecosystems and merit study in their own right. It is now recognized that the influence of parasites extends beyond the host individual to the population and community level and may affect ecosystems through differential effects on host species and their effects on keystone species. However, it is becoming less acceptable to collect large numbers of hosts for single objective studies. Thus, the need for coordination among researchers to maximize the data collected, while always desirable, has become more and more imperative. Indeed, the biological and parasitological data obtained in collaborative studies frequently complement each other.

Unfortunately, proper collection of parasites requires time and expertise. As a result, collection of parasites cannot be accomplished effectively as an “add on” activity

by researchers studying other aspects of the host. This inevitably results in poor, often unidentifiable, specimens and questionable counts. If parasites are to be included as part of a larger study a parasitologist, or someone trained in the appropriate techniques, should be involved in the planning phases and sufficient time and resources need to be allocated to do the work.

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## APPENDIX I

Anatomical locations of Protozoan (P), Digenean (D), Nematode (N), Leech (L), Louse (LO), Tick (T), Mite (M), Flea (F) and Pentastome (PE) infections in birds, exclusive of the digestive tract. Modified after McDonald (1974, 1981), Doster and Goater (1997) and Clayton and Walther 1997).

| LOCATION | P | D | N | L | LO | T | M | F | PE |
|---|---|---|---|---|---|---|---|---|---|
| Body surface | | X | | X | | X | X | X | |
| Feathers | | | | | X | | X | X | |
| Eye | | X | X | | | | | | |
| Muscle | X | X[1] | X[1] | | | | | | |
| Subdermis | | X | X | | | | X | | |
| Joints | | | X | | | | | | |
| Eye | | X | X | | | | | | |
| Nasal veins | | X | | | | | | | |
| Nasal sinus | | X | | X | | | X | | |
| Mouth, Tongue | | X | X | | X | | | | |
| Brain | | | X | | | | | | |
| Body cavity | | | X | | | | | | |
| Trachea | | X | X | | | | X | | |
| Lungs Bronchi | | X | X | | | | X | | |
| Air sacs | | X | X | | | | X | | X |
| Heart Aorta Veins | | | X | | | | | | |
| Dorsal aorta | | | X | | | | | | |
| Mesenteric veins | | X | | | | | | | |
| Renal veins | | X | | | | | | | |
| Blood/Lymph | X | | X | | | | | | |
| Kidney | X | X | | | | | | | |
| Ureters | X | X | | | | | | | |
| Oviduct | | X | | | | | | | |

## APPENDIX II

Anatomical locations of Protozoan (P), Digenean (D), Cestode (C), Acanthocephalan (A) and Nematode (N) parasites infecting the digestive tract of birds. Modified, in part, after McDonald (1974, 1981) and Doster and Goater (1997).

| LOCATION | P | D | C | A | N |
|---|---|---|---|---|---|
| Mesenteries (external) | | | | | X |
| Esophagus | | X | | | X |
| Proventriculus | | X | | | X |
| Gizzard | | | X | | X |
| Crop / Stomach[1] | X | | | | X |
| Intestine (total) | X | X | X | X | X |
| Caeca | X | X | X | | X |
| Cloaca | | X | X | | |
| Bursa of Fabricius | | X | | | |
| Liver | | X | | | |
| Gall bladder and Bile duct | | X | | | |
| Pancreas and Duct | | X | | | |

## APPENDIX III
## LIST OF SUPPLIERS (CANADA)

### GENERAL SUPPLIES / EQUIPMENT

Most of the equipment, supplies and reagents necessary are common laboratory items and can be purchased from an number of companies. This is a listing of the major suppliers; there are many others who can provide these items.

**Anachemia Science**, 255 Rue Norman, Ville St. Pierre, Quebec, H8R 1A3.

Tel: 800-361-0209
Fax: 800-438-9777
E-mail: info@anachemia.com
Website: http://www.anachemia.com

**Canadawide Scientific**, 2300 Walkley Road, Unit 414, Ottawa, Ontario, K1G 6B1.

Tel: 800-267-2362
Fax: 800-814-5162
E-mail: cws@canadawide.com

**Fisher Scientific**, 112 Colonnade Road, Nepean, Ontario, K2E 7L6.

Tel: 800-234-7437
Fax: 800-463-2996
Website: http://www.fishersci.ca

**VWR Canlab (Canada)**, 2360 Argentia Road, Missauga, Ontario, L5N 5Z7.

Tel: 800-932-5000
Fax: 800-668-6348
Website: http://www.vwrsp.com

### DISSECTING INSTRUMENTS

An excellent range of high quality dissection instruments is available from:

**Fine Science Tools**, 202-277 Mountain Highway, North Vancouver, British Columbia, V7J 3P2.

Tel: 800-665-5355
Fax: 800-665-4554
E-mail (orders): canada@finescience.com
Website: http://www.finescience.com

## APPENDIX IV
## LIST OF SUPPLIERS (CANADA)

### STAINS AND HISTOLOGICAL REAGENTS

Stains and reagents can be purchased from some of the companies listed in Appendix III (e.g. Fischer); additional sources are listed below.

**BDH Inc.**, 350 Evans Avenue, Toronto, Ontario, M8Z 1K5.

Tel: 800-268-0310
Fax: 800-551-7052
Website: http://www.bdhinc.com

**Sigma-Aldrich Canada Ltd.**, 2149 Winston Park Drive, Oakville, Ontario, L6H 6J8.

Tel: 800-565-1400
Fax: 800-265-3858
E-mail: canada@sial.com
Website: http://sigald.sial.com/canada

## APPENDIX V
## SALINES, FIXATIVES, CLEARING / MOUNTING MEDIA AND STAINS

The following are some of the more routinely used chemicals.

### SALINE

**0.85% Saline**

Stock:
Mix 0.85 gm NaCl per 100 ml distilled water.

Working Solution:
Use undiluted.

### FIXATIVES

**Formalin**

Stock:
Available commercially as a 37% solution. This is considered as 100% for dilution purposes. Also available commercially in diluted and buffered solutions.

Working Solution:
Usually 4-10% by volume.

**Bouin's Fixative** (Ash and Orihel, 1991). DANGER: Picric Acid..

Stock:
Mix 25 ml formaldehyde (37% USP) and 75 ml saturated aqueous solution picric acid.
NOTE: picric acid is dangerous - use caution.

Working Solution:
Add 5ml glacial acetic acid to100 ml stock solution just prior to use.

**Acetic Acid-Formalin-Alcohol** (AFA or FAA) (Ash and Orihel,1991).

Stock:
Mix 45 ml distilled water, 10 ml formaldehyde, 5 ml glacial acetic acid, and 50 ml of 95% ethanol.

Working Solution:
Use undiluted.

**Schaudinn Fixative** (Humason, 1972). DANGER: Mercuric Chloride.

Stock:
Mix 66 ml saturated aqueous mercuric chloride, 33 ml 95% ethanol and 5-10 ml glacial acetic acid.

Working Solution:
Use undiluted; post treat with Lugol's Iodine for mercuric chloride.

**Lugol's Iodine** - for Schaudinn postreatment (Ash and Orihel, 1991).

Stock:
Dissolve 10 gm KI in 100 ml distilled water.
Add 5 gm iodine crystals and shake until most of the crystals dissolve.
Store in brown bottle; shelf life about four weeks.

Working solution:
Use 1 part Lugol's to 5 parts distilled water.

## CLEARING / MOUNTING MEDIA

**Berlese Fluid** (Humason, 1972).

Stock:
Dissolve 5 ml dextrose syrup in 10 ml distilled water and 3 ml glacial acetic acid . Add 8 gm gum arabic and wait until dissolved (>1 week) then add 75 gm chloral hydrate.

Working Solution:
Use undiluted.

**Hoyer's Medium** (Ash and Orihel, 1991).

Stock:
Mix 30 gm gum arabic, 20 ml glycerol, 50 ml distilled water, and 200 gm chloral hydrate.
Filter through gauze.

Working Solution:
Use undiluted.

**5% Glycerine in 70% Ethanol**

Stock:
Mix 5 ml glycerol in 95ml of 70% ethanol

Working Solution:
Use undiluted.

**Lactophenol** (Ash and Orihel, 1991).

Stock:
Mix 20ml glycerine and 10 ml each of lactic acid, melted phenol crystals and distilled water.

Working Solution:
Use undiluted.

**STAINS**

Giemsa, Acetocarmine, Ehrlich's Haematoxylin and Delafield's Haematoxylin Stains are available commercially. Most can also be made in the laboratory. See Ash and Orihel (1991) or similar source for details.

**Schneider's Carmine** (Ash and Orihel, 1991).

Stock:
Add 45 ml glacial acetic acid and 55 ml distilled water in a flask.
Add 5 g carmine powder.
Boil 15 minutes, cool and filter.

Working Solution:
Use dilute in 70% ethanol; add drop by drop until alcohol is a dark pink.

**Van Cleave's Haematoxylin** (Ash and Orihel, 1991).

Stock:
1 ml Delafield's and 1 ml Ehrlich's haematoxylins in 100 ml distilled water.
Add 6 gm potassium ammonium sulfate and mix well.

Working Solution:
Use undiluted.

## PERMANENT MOUNTING MEDIA

Canada balsam and a variety of synthetic media e.g. Permount are available commercially. Glycerine jelly, used for semi permanent mounts of nematodes, is also available commercially.

**APPENDIX VI**
**SAMPLE EXAMINATION SHEET**
(Customize as Required)

HOST _____ HOST NUMBER _____

DATE _____ AGE _____ SEX _____

LOCALITY _____

COLLECTOR _____

WEIGHT _____ LENGTH_____ TARSUS_____ CULMEN _____

NOTES

| **Location** | **Parasites** | **# Vials** | **# Frozen** |
|---|---|---|---|
| Air Sacs | | | |
| Body Cavity | | | |
| Trachea | | | |
| Eosphagus | | | |
| Proventriculus | | | |
| Gizzard | | | |
| Intestine | | | |
| Caecum | | | |
| Bursa | | | |
| Gall Bladder | | | |
| | | | |
| | | | |
| | | | |

## APPENDIX VII
## STAINING PROCEDURES MACROPARASITES - AN OVERVIEW

Standard Stains:

A number of standard stains are can be used in the preparation of whole mounts. Acetocarmine and acetic acid alum carmine are examples of progressive stains and are the easiest to use for routine work.

Schneider's carmine and Ehrlich's, Delafield's or Van Cleave's haematoxylin also give good results but require extra steps. These overstain the specimens and the excess removed gradually to the desired intensity then fixed in the tissue. See Differentiation below.

Washing:

In each case, specimens must be placed in 70% ethanol for a few days prior to staining to remove any fixative that could interfere with the staining process. See the Tips section below for other useful suggestions.

Hydration:

Most of the stains are water based and require that the ethanol be removed and replaced with water before the specimens are placed in them. This is referred to as hydration and is accomplished by placing specimens in successively more dilute solutions of ethanol (70%, 50%, 30% ethanol) and distilled water for brief periods (2-10 minutes depending on the size of the specimens). Schneider's carmine (which is used in 70% ethanol) is an exception and specimens can be placed in the stain directly from 70% ethanol.

Staining:

Carmine stains are used in diluted form and some testing may be necessary to determine the optimum concentration and time. Typically 5 drops per 50 ml of distilled water is sufficient and the specimens are stained for 12 -24 hours (or longer).

Haematoxylin stains are normally used in concentrated form. Except for Van Cleave's haematoxylin, they are thick and dark and not recommended for small specimens which are easily lost. (If used for small specimens, dilute with distilled water before removing the specimens to make them easier to see. DO NOT use tap water). Specimens are normally stained for a few minutes then processed. See Ash and Orihel (1991) or any standard tissue techniques text (e.g. Humason 1972) for procedures.

Dehydration:

This process involves removal of water from the tissues by transferring the specimens through increasingly concentrated alcohol solutions to absolute ethanol. Two washes with absolute ethanol are required to ensure all traces of water are removed before the specimens are placed in solvent for clearing.

Differentiation:

Specimens stained in haematoxylin or in Schneider's carmine are normally overstained and the excess must be removed. This process is called differentiation and is accomplished by placing the specimens in a 1% solution of HCl in 70% ethanol after the 70% step in the dehydration sequence. When sufficient stain has been removed, the specimens are placed in a dish of clean 70% ethanol to remove the acid and stop the process. Ash and Orihel (1991) recommend adding a few drops of saturated aqueous sodium carbonate or lithium carbonate to the neutralize the acid. Following differentiation all specimens stained in haematoxylin need to be "blued". This is done by transferring the specimens to a dish of 1% $NH_4OH$ in 70% ethanol for few minutes. This precipitates the stain and turns the specimen blue. Excess 1% $NH_4OH$ is removed in fresh 70% ethanol and the dehydration process is resumed through the 95% and absolute ethanol steps outline above.

Clearing:

This process involves the use of a solvent (xylene is the common one) to replace the alcohol in the specimens. This renders much of the specimen transulcent and provides a means for the mounting medium to penetrate the tissue. Two successive washes are required before the specimens are ready to mount.

Mounting:

A variety of mounting media are available. Canada balsam is the best and should be used for archival material. It has some drawbacks; it is expensive and takes a long time to dry. Several synthetic media are available commercially. These are less expensive, dry faster but in some cases are prone to deterioration over time. All slides should be properly labelled, and stored in boxes.

Tips:

1. Transfer as little fluid as possible between steps.
2. Use clean pipettes to transfer small specimens between alcohol and xylene and between the xylene steps. Don't mix pipettes to avoid transfer of water to clearing solvents.
3. Use **only** glass dishes for solvents.
4. Small specimens, particularly those fixed in hot ethanol, have a tendency to stick to glass dishes in lower concentrations of alcohol. If this occurs, use plastic dishes for these steps.
5. An electric engraving tool, available at most hardware outlets, is particularly useful for permanently marking slides.
6. If possible use #1 thickness cover slips.

## APPENDIX VIII
## ACETOCARMINE STAINING PROCEDURE FOR MACROPARASITES

The procedure outlined below is for acetocarmine. Consult Ash and Orihel (1991) for procedures involving Schneider's carmine and the haematoxylin stains.

Hydration and Staining:

Times for alcohol and xylene steps vary from 5-15 or more minutes depending on the size of the specimens. A good rule of thumb is to allow at least 5 minutes after the specimens sink.

Specimens are normally stored in 70% ethanol. The hydration process described here begins at that point.

70% Ethanol
50% Ethanol
30% Ethanol
Distilled water
Acetocarmine (5-drops / 50ml of distilled water - 12 -24 hours)

Dehydration, Clearing and Mounting:

30% Ethanol
50% Ethanol
70% Ethanol
95% Ethanol
Absolute Ethanol
Absolute Ethanol
Xylene
Xylene

Mount

## APPENDIX IX
## STAINING TECHNIQUES FOR MICROPARASITES: SECTIONS AND SMEARS

**Sections:**

Sectioned tissue specimens fixed to microscope slides are first dewaxed and hydrated prior to staining. Normally sections are stained in haematoxylin and counterstained in eosin. The following protocol is for Ehrlich's haematoxylin. Times for each step and for staining are approximate. Times required for other stains may vary.

Dewaxing, Hydration and Staining:

| | |
|---|---|
| Xylene | (2 minutes;discard after use) |
| Xylene | (2 minutes) |
| Xylene | (2 minutes) |
| Absolute Ethanol | (2 minutes) |
| Absolute Ethanol | (2 minutes) |
| 95% Ethanol | (2 minutes) |
| 70% Ethanol | (2 minutes) |
| 50% Ethanol | (2 minutes) |
| 30% Ethanol | (2 minutes) |
| Distilled water | (2 minutes) |
| Stain | (5-10 minutes) |

Dehydration, Differentiation, Blueing, Counterstaining Clearing and Mounting.
The solutions used in the earlier steps, except for the discarded xylene, can be reused.

| | |
|---|---|
| Distilled water | (Quick rinse) |
| 30% Ethanol | (2 minutes) |
| 50% Ethanol | (2 minutes) |
| 70% Ethanol | (2 minutes) |
| 1% HCl / 70% Ethanol[1] | |
| 70% Ethanol | (2 minutes) |
| 1% $NH_4OH$ / 70% Ethanol[2] | |
| 70% Ethanol | (2 minutes) |
| 95% Ethanol | (2 minutes) |
| Eosin[3] | (1-2 minutes) |
| 95% Ethanol[4] | |
| Absolute Ethanol | (2 minutes) |
| Absolute Ethanol | (2 minutes) |
| Xylene | (2 minutes) |
| Xylene | (2 minutes) |

Mount

[1] Differentiation: examine periodically under a microscope; cytoplasm should be colourless, nuclei pink.
[2] As required; 2-4 minutes is normally sufficient.
[3] Saturated solution of eosin in 95% ethanol.
[4] 95% ethanol will remove eosin quickly; test and adjust times for personal preference.

Gut or Tissue Smears:

Staining procedures for sections and smears is similar to that for whole mounts. The only difference is the type of glassware needed. Some trial and error is necessary to establish optimum differentiation, blueing and counterstaining times for sections and smears.

Fresh material is smeared on a microscope slide and fixed in Schaudinn's, or some other fixing fluid, as soon as they are made. Do not let the smear dry. Fix for an 40-60 minutes then wash in Lugol's Iodine in 70% ethanol for 30 minutes. Hydrate from 70%, through 50% and 30% ethanol to distilled water. The smears are stained in haematoxylin and processed as described for sections. Counter staining in eosin is optional.

## APPENDIX X
## EXAMPLE OF A DATA SHEET FOR A DIGENEAN

(Customize as Required)

**IDENTIFICATION:**

**HOST SPECIES:_____HOST CODE NUMBER:**

| | |
|---|---|
| **Slide No.** | |
| **Body Length** | |
| **Body Width** | |
| **Oral Sucker L** | |
| **Oral Sucker W** | |
| **Prepharynx L** | |
| **Pharynx L** | |
| **Pharynx W** | |
| **Esophagus L** | |
| **Acetabulum L** | |
| **Acetabulum W** | |
| **Distance Anterior** | |
| **Anterior Testis L** | |
| **Anterior Testis W** | |
| **Posterior Testis L** | |
| **Posterior Testis W** | |
| **Cirrus Sac L** | |
| **Cirrus Sac W** | |
| **Ovary L** | |
| **Ovary W** | |
| **Egg L** | |
| **Egg W** | |
| **Notes** | |
| | |
| | |
| | |

## APPENDIX XI
## STAINING TECHNIQUES FOR BLOOD SMEARS

Blood Smears:

Giemsa stain is the preferred stain for blood films (Ash and Orihel,1991) and can be obtained commercially as a stock solution. Buffered water for washing is recommended (see Ash and Orihel, 1991) but running tap water can be substituted.

Proceedure (from Ash and Orihel,1991).

1. Fix smears in 100% ethanol or methanol for 30 seconds. Allow to dry.
2. Stain smears in Giemsa:
   1:20 dilution 20 minutes.
   1:50 dilution 45 minutes.
3. Wash under neutral buffered water or running tap water.
4. Dry smears in a vertical position.
5. Mount when dry.

## APPENDIX XII
## TAXONOMIC REFERENCES FOR THE IDENTIFICATION OF ENDOPARASITES (COCCIDIA AND HELMINTHS) OF BIRDS

***MICROPARASITES***
**Coccidia**:

Todd and Hammond (1971): Descriptions of oocysts from Anseriformes, Passeriformes and Galliformes.

***MACROPARASITES***
**Digenea**
Yamaguti (1971): keys to genera and species lists.
Schell (1970): keys to genera only.
Schell (1985): keys to genera only.
Skrjabin (1964): keys to genera; species lists and keys to species for many of the smaller genera.

**Cestoda**
Schmidt (1970): keys to genera.
Schmidt (1986): keys to genera and species lists.
Khalil et al. (1994): keys to genera only.

**Nematoda**
Yamaguti (1961): keys to genera and species lists.
Anderson, Chabaud and Willmott (1974-1983): keys to genera only. This series consists of 10 volumes (each by a specialist) over the nine year period.

Volume 1 Terminology and keys to Subclasses, Orders and Superfamilies
Volume 2 Superfamily Ascaroidea.
Volume 3 Order Spirurida (in three parts).
Volume 4 Superfamily Oxyuroidea.
Volume 5 Superfamily Metastrongyloidea.
Volume 6 Superfamilies Cosmocercoidea, Seuratiodea, Heterakoidea, Subuluroidea.
Volume 7 Superfamily Strongyloidea.
Volume 8 Superfamilies Ancylostomatoidea and Diaphanocephaliodea.
Volume 9 Superfamilies Rhabditioidea, Dioctophymatioidea, Trichinelloidea and Muspiceoidea.
Volume 10 Superfamily Trichostrongyloidea and an index to the series.

**Acanthocephala**
Yamaguti (1963): keys to genera and species lists.
Petrochenko (1971): keys to genera; many genera with keys to species.

**Additional Sources**

In addition to the references listed above, three series of Russian monographs; Essentials of Trematodology, Essentials of Cestodology and Essentials of Nematology are very useful for the identification of avian helminths. Each monograph deals with a single family or a small number of families and contains keys to the genus and species level. Detailed descriptions of each species (usually including the original description, supplemented with additional data) are provided. A list of these can be found under the entries for Skrjabin, K. I. et al. in McDonald (1969). Several volumes have been translated into English.

A number of other useful reference books and monographs on the parasites of birds have been published by authors in Eastern Europe and the former Soviet Union. Several of these have been translated into English as well.

## APPENDIX XIII
## TAXONOMIC REFERENCES FOR THE IDENTIFICATION OF ECTOPARASITES

### HIRUDINEA
Sawyer (1972), Pennak (1989), and Davies (1991) provide keys to the genera and species of North American freshwater leeches.

### ARTHROPODA GENERAL
Furman and Catts (1982): provide keys to genera and species of a number of parasitic insects (Mallophaga, Anoplura, Diptera, Siphonaptera) and chelicerates (Acarina). There are many references to older monographic works on several of these orders, including a number specific to North America. Among these are extensive studies on fleas with keys to the family, genus and species levels. More recent references are listed below. Additional titles are listed in Bell and Rhodes (1994).

### ACARINA
Krantz (1978): keys to family level for ticks and mites.
McDaniel (1979): keys to genera of ticks and mites.
Sonenshine (1993): keys to genus and species of ticks.

### SIPHONAPTERA
Lewis et al. (1988): restricted to species of the Pacific Northwest, U.S.A.
Hopkins and Rothschild (1966-1987): a review of fleas of the world, based on the Rothschild collection in the British Museum (Natural History).

### MALLOPHAGA
Furman and Catts (1982) provide keys to the families of Amblycera and Ischocerca found in North America.

## APPENDIX XIV
## TAXONOMIC REFERENCES FOR THE IDENTIFICATION OF HAEMATOZOA

Garnham (1966): A major monograph on blood parasites.

Valkiunas (1997): A recent monograph on avian haemosporidia (in Russian but with an English summary and index).

Greiner and Bennett (1975): A pictorial guide to species of *Haemoproteus, Leucytozoon* and *Trypanosoma* of birds (microfiche).

Greiner, Bennett, Laird and Herman (1975): A pictorial guide and keys to species of *Plasmodium* of birds (microfiche).

There are also a number of extensive bibliographies and host-parasite lists of blood inhabiting protozoa including those of Herman et al. (1976), Bennett et al. (1981), Bennett et al. (1982), Bishop and Bennett (1992) and Bennett et al. (1989).