ECOLOGICAL MONITORING AND ASSESSMENT NETWORK (EMAN)

PROTOCOLS FOR MEASURING BIODIVERSITY:

PARASITES OF AMPHIBIANS AND REPTILES

by

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INTRODUCTION

Why study parasites of amphibians and reptiles (herps, hereafter)?

Parasites comprise a vast diversity of organisms that are specifically adapted to living in, or on, another living organism (the host). Over 50% of described organisms can be classified as parasites (Price, 1980). They are metabolically dependent on the host for survival, and feed and reproduce at the host's expense. The parasites of amphibians and reptiles comprise a diverse array of phylogenetically distinct taxa. In this report we first review the advantages to studying herps and their parasites, review the diverse parasite taxa one can expect in herps, and then provide detailed sampling and quantification procedures for the parasites of reptiles and amphibians. Our focus is on the herps of North America, especially those encountered in Canada.

When the terms "biological diversity", "biodiversity crisis" or "conservation biology" are used, one group typically comes to mind: amphibians and reptiles. This is because many amphibian and reptile species around the world have been documented to be undergoing alarming population declines, range reductions and, even extinctions (e.g. Wake, 1991; Blaustein et al., 1994a, b; Blaustein and Wake, 1995; Gibbons et al., 2000). Most amphibians respire cutaneously and have a complex life cycle with an aquatic larvae and a rapid transformation, or metamorphosis, to a more terrestrial adult. Distinct morphological, behavioural and ecological changes accompany amphibian metamorphosis (Wilbur, 1980; Werner, 1986). Thus, this biphasic life cycle as well as their aquatic respiratory constraints,

has led to the notion that amphibians are “sentinels” or “canaries in the coal mine”; in other words, that amphibians are indicators of global environmental degradation (Blaustein and Wake, 1995; Morrell, 1999). The factors that may be responsible for amphibian and reptile declines range from habitat destruction and degradation, introduction of invasive species, increased ultraviolet radiation, pollution, infectious disease and helminth parasites (Wake, 1992; Blaustein, 1994;; Blaustein et al., 1994a, b; Blaustein and Wake, 1995; Morrell, 1999; Gibbons et al., 2000; Kiesecker et al., 2001, but see Pechmann et al., 1991; Pechmann and Wilbur, 1994). Recently, the latter hypothesis, that parasites may be responsible for some amphibian declines, has received considerable attention. The recent interest in parasites in amphibians probably stems from the provocative papers by Johnson et al. (1999, 2001) and the popularized account by Souder (1999). Johnson et al. (1999, 2001) offer compelling experimental evidence that a trematode parasite, *Ribeiroia* sp. is responsible for the dramatic limb deformities observed in certain amphibian populations in several areas of North America (see also Sessions and Ruth, 1990). Johnson et al. (1999) conclude by stating: “These questions, coupled with the exteme mortality and abnormality rates observed in this study, call for increased research focus on parasitic infection and its effects on amphibian host populations”.

Herp-parasite systems have figured prominently in such fields as ecology, evolutionary biology, and animal behaviour. Herps afford excellent model systems for field and laboratory studies. Thus, because many herps are long-lived, have life histories with two distinct niches and display an astonishing variety of life histories

and reproductive modes (Werner, 1986), herps have been widely used as model organisms in understanding life history evolution, evolutionary ecology and animal behaviour, (e.g. Huey et al., 1983; Ryan, 1985; Semlitsch and Gibbons, 1985; Hairston, 1987; Seigel et al., 1987; Gibbons, 1990; Wilbur 1980, 1987; Seigel and Collins ,1993; Whiteman, 1994; Brodie and Brodie, 1999; Shine and Bonnet, 2000). We argue that no other host group offers the rich advantages of experimental manipulations in the field and in the laboratory, or the diversity of life histories, or range of habitats, as do reptiles and amphibians. These advantages have enabled parasitologists and evolutionary ecologists to use selected herp-parasite systems as an introduction to teaching parasitology (e.g. Smyth and Smyth, 1980), and to address several controversial areas in biology.

The first concerns the evolution of virulence and parasite-mediated natural selection (see review by Goater and Holmes, 1997). Two of the most comprehensive studies in our view are the long-term investigations of the leech-trypanosome-red-spotted newt (Mock and Gill, 1984; Gill and Mock, 1985) and the fence lizard-malaria studies conducted by Schall (e.g. Schall, 1983; 1990).

Another controversy is that parasites have been suggested to influence mate choice decisions and to be agents of parasite-mediated sexual selection (Hamilton and Zuk, 1982). The lizard malaria experiments involving female choice and male colour patterns, provide an example and a possible alternative to the Hamilton and Zuk hypothesis (e.g. Schall, 1990).

A third example addresses the hypothesis that parasites may regulate the abundance of host populations through parasite-induced mortality of heavily

infected hosts (May and Anderson, 1978). The experimental studies of the lung nematode, *Rhabdias bufonis* in European toads provide an example. In this system *R. bufonis* reduces the growth, physical performance and survival of juvenile toads (Goater and Ward, 1992; Goater et al., 1993; but see Goater, 1994).

Fourth, the first manipulative experimental study conducted on vertebrates to examine the hypothesis that parasites mediate competitive interactions between free-living animals and thus, influence community structure (see Minchella and Scott, 1991) was conducted on frogs and the pathogenic fungus, *Saproglenia ferax* (Kiesecker and Blaustein, 1999).

In addition, herp-parasite systems have also provided unique opportunities of addressing concepts in parasite population biology (e.g. Jarroll, 1979; Tinsley, 1989; Goater, 1992, Goater and Vandenbos, 1997; Wetzel and Esch, 1996a, 1997; Zelmer et al., 1999), and parasite community ecology (e.g. Goater et al., 1987; Aho, 1990; Janovy et al., 1992; Fontenot and Font, 1996; McAlpine, 1997a; Goldberg et al., 1998). Phylogenetic studies of several herp-parasite systems have also provided valuable insight into host-parasite biogeographic patterns and the nature of coevolutionary relationships between hosts and between hosts and their parasites (e.g. Ernst and Ernst, 1980; Platt, 1992).

Finally, many biologists overlook the potential for using the presence or absence of helminth parasites as indicators of trophic interactions and other aspects of their host's biology. Many helminth (worm) parasites have complex life cycles with intermediate hosts, and typically exploit food web relationships,

particularly predator-prey interactions for transmission. Thus, many parasites can be used as “biological tags”, and provide valuable information on the feeding habits and patterns of habitat use of their vertebrate hosts. Information provided by parasites is particularly valuable for vertebrates which are secretive, nocturnal and/or fossorial; certainly the majority of amphibians and reptiles (Goater, 1990). Thus, parasites can be valuable as a means of tracing food-web relationships and food-web structure, as well as reflecting the biodiversity of their habitat (Marcogliese and Cone, 1997; Brooks and Hoberg, 2000; Marcogliese, 2001). This is especially important for herps, as many occupy pivotal positions in food webs, functioning as important predators in food chains or as significant prey species in others. Herps can also be very abundant and comprise significant components of vertebrate biomass in certain terrestrial and freshwater ecosystems (Hairston, 1987; Heyer et al., 1994). Parasites can also be excellent indicators of environmental contaminants and stress, especially in aquatic ecosystems (MacKenzie et al., 1995). This can be especially important for herp-parasite systems, since the hosts themselves can be valuable bioindicators (e.g. Bonin et al., 1995). In all of these cases it is critical to understand several aspects of host ecology and parasite phylogeny, host specificity and life cycle dynamics.

TYPES OF HERP PARASITES: AN OVERVIEW

Microbial and Protistan Parasites

Herps are host to an astonishing diversity of microparasites and macroparasites. The microparasites of herps include viruses, bacteria and fungi as

well as the eukaryotic, unicellular Protista. These microbes and protists are capable of replication and rapid development in the host, and several microbes are causative agents of serious infectious diseases of wild populations of amphibians and reptiles. It should be recognized that microbes are a significant component of biological diversity and should not be ignored, especially given their potential for severe pathogenicity in herps. For example, chytridiomycosis (caused by a fungus) and iridoviruses, such as *Ranavirus* sp. have both been responsible for causing catastrophic amphibian mortalities (e.g. Daszak et al., 1999; Morell, 1999; Bollinger et al., 1999). Perhaps the most well-known bacterial disease of amphibians is so-called 'red leg', caused by the bacterium, *Aeromonas hydrophila*. This bacterium species has been implicated in causing certain amphibian mass mortalities and local extinctions (e.g. Nyman, 1986; Carey, 1993). Recently, Taylor et al. (1999) found that the pesticide malathion increased disease susceptibility of Woodhouse`s toad, *Bufo woodhousi*, to *A. hydrophila,* stressing the potential of interactive or synergistic effects in causing amphibian population declines (see also Kiesecker and Blaustein, 1995; Kiesecker et al., 2001). Another microbial pathogen, the fungus *Saprolegnia ferax,* is the causative agent of massive amphibian embryo mortality in the Pacific Northwest (Kiesecker and Blaustein, 1997). An intriguing aspect of this host-parasite system is that some anuran hosts (e.g. cascades frog) experience high mortality from the fungus, while other frog species (e.g. Pacific treefrog) appear unaffected. Differences in life history traits, especially egg-laying behaviour are important determinants of the infection rate by *S. ferax* (Kiesecker and Blaustein, 1997). An elegant experiment demonstrated that this pathogenic

fungus can greatly affect these two frog species' competitive interactions, and can ultimately influence amphibian community structure (Kiesecker and Blaustein, 1999).

Another large and diverse group of microparasites are the eurkaryotic, single-celled animals classified in the Kingdom Protista. Herps can be host to a range of coccidian intracellular blood and intestinal parasites, as well as to blood-dwelling, extracellular flagellates, and to ciliates.

The Apicomplexa is well-represented in herps. Familiar examples include *Plasmodium* spp., a common genus of intracellular parasites in the blood of lizards, and the causative agent of malaria (e.g. Schall, 1983; 1990). Indeed, approximately half of the described species of *Plasmodium* are parasites of lizards (Schall, 1990). Coccidian parasites, especially hemogregarines such as *Hepatozoon* spp., *Haemogregarina* spp., and *Babesiosoma* spp. are intracellular blood parasites of aquatic herps, including frogs (e.g. Barta and Desser, 1984; 1986; Smith et al., 2000), and turtles (e.g. Siddall and Desser, 1991). Other coccidians, such as *Eimeria* spp., are common intestinal parasites of herps, including anuran amphibians (e.g. Upton and McAllister, 1988), lizards (e.g. Duszynski, 1969; Telford, 1998; Modry et al., 2000), and turtles (e.g. McAllister et al., 1991a). In addition, hemoflagellates such as *Trypanosoma* spp. infect a wide range of aquatic herps, including frogs (e.g. Barta and Desser, 1984; Martin and Desser, 1991), salamanders (e.g. Woo et al., 1980; Woo and Bogart, 1986; Goater, 2000) and turtles (e.g. Woo, 1969a). Many of the blood-dwelling apicomplexan and flagellated protists require hematophagous leech vectors for transmission from host

to host. Mosquitoes and other dipteran flies may also act as vectors for apicomplexan and kinetoplastid parasites. For example, *Hepatozoon* spp. use hematophagous arthropods as vectors (Smith, 1996).

Other protists infecting primarily anuran amphibians include the large, flagellated intestinal parasites such as *Opalina* spp. and the ciliate, *Nyctotherus cordiformis* (e.g. Bolek and Coggins, 1998a). Some amphibians can also be opportunistic hosts for ciliated protists usually considered as fish ectoparasites, including *Ichthyophthiurus multifiliis* and *Trichodina* spp. (Gleeson, 1999).

Myxozoan Parasites

The enigmatic and complex, spore-forming Myxozoa are known almost entirely as fish parasites (e.g. *Myxobolus*, *Kudoa*); however, some myxozoans infect certain herps. For example, *Myxidium serotinum* has been reported infecting the gall bladder of several species of frogs and salamanders (e.g. McAllister and Upton, 1987). *Chloromyxum salamandrae* infects certain plethodontid salamanders, including *Desmognathus brimleyorum* and *Eurycea* spp. (McAllister et al., 1995a).

Helminth Parasites

Helminths include the so-called “worm” parasites, actually a diversity of animals which includes the monogenean and digenean trematodes (flukes or flatworms), the cestodes (tapeworms), the nematodes (round worms) and the acanthocephalans (thorny-headed worms).

All three parasitic classes of the Phylum Platyhelminthes are represented in herps. Thus, some amphibian species are host to monogenean trematodes, which have a direct life cycle featuring a free-swimming aquatic larval stage. Some common genera of monogeneans infecting amphibians include *Sphyranura* (ectoparasites that infect the skin and external gills of some aquatic salamander species such as the mudpuppy, *Necturus maculosus,* and *Polystoma, Pseudodiplorchis* and *Iagotrema* (endoparasites of the urinary bladder of frogs, toads and freshwater turtles, respectively) (see Prudhoe and Bray, 1982). *Neopolystoma* spp. infect the conjunctival sac of turtles (Platt, 2000)

Most of the other worm parasites have complex life cycles with intermediate hosts; transmission to the definitive host (host in which reproduction of the parasite occurs) is often dependent upon ingestion of intermediate hosts containing infective larval worms. Given their importance as prey and/or as predators in food webs, herps can be host to a variety of larval and adult helminth parasite species. Host habitat and dietary preferences play critical roles in the transmission of helminth parasites of herps (see Goater et al., 1987; Aho, 1990; McAlpine, 1997, for examples). Specific references relevant to the helminth parasite diversity of anuran species found in Canada include Coggins and Sajdak

(1982); Muzzall (1991a); McAllister et al. (1995a), Yoder and Coggins (1996), McAlpine (1997a), McAlpine and Burt, (1998), Gillilland and Muzzall, (1999) for frogs in the Family Ranidae; Bursey and Goldberg, (1998), for toads in the Family Bufonidae; Bolek and Coggins (1998a), Yoder and Coggins (1996) for tree frogs in the Family Hylidae. For specific references to the helminths of Canadian species of salamanders, see Bursey and Schibli (1995), Bolek and Coggins (1998b) (Plethodontidae); Muzzall (1991b) (Salamandridae); Bolek and Coggins (1998b) (Ambystomatidae). In terms of the helminth diversity of Canadian reptiles, Rau and Gordon (1980) provide information on snakes in the Family Colubridae, Goldberg and Bursey (1991) present data on anguid lizards, and Esch and Gibbons (1967), Ernst and Ernst (1977, 1980) provide data on turtles.

Before beginning a parasite diversity study, investigators should obtain these references in order to determine the types of helminth parasites one may find in an amphibian or reptile species collected in Canada. It should also be recognized that older references and herp host-parasite checklists are also valuable (e.g. Rankin, 1937; Yamaguti, 1958). Specific taxonomic references, as well as published keys will also be essential in order to determine the specific identification (e.g. Baker, 1978; Kennedy, 1981; Anderson, 1992).

Flatworm parasite species within the Class Trematoda (or Subclass Digenea, by some authors), commonly called flukes, are described from a variety of amphibians and reptiles. Because molluscs (especially aquatic snails) are essential as first intermediate hosts for trematodes, aquatic amphibians are more commonly infected than are more terrestrial amphibians and reptiles. Many adult

trematodes live within the small intestine of herps, but several are site specific to other regions. To stress this point, consider two commonly encountered genera of amphibian trematodes: *Halipegus* and *Haematoloechus*. Adults of *Halipegus occidualis* live under the tongue of green frogs, *Rana clamitans*, while *Halipegus eccentricus* lives in the eustachian tubes of the same frog host (see Goater et al., 1990a, b; Wetzel and Esch, 1996a, b). There is a degree of controversy surrounding the nomenclature of the genus *Halipegus* (McAlpine and Burt, 1998; Zelmer and Esch, 1999). *Haematoloechus* spp. adults are strictly lung parasites, infecting primarily ranid frogs, often showing pronounced geographic variation in morphology (see Kennedy 1980; 1981). Both genera rely on frog predation of dragonfly and damselfly naiad second intermediate hosts, in which infective metacercariae can be found (Goater et al., 1990, Wetzel and Esch, 1996b; Zelmer and Esch, 1998). Other common trematode genera are those found in the urinary bladder of frogs (e.g. *Gorgoderina* spp., McAlpine and Burt, 1998), the small intestine of salamanders (e.g. *Brachycoelium* spp., Goater et al., 1987), and the colon of various salamanders and frogs (e.g. *Megalodiscus* spp., Joy and Pennington, 1998).

Amphibians can also act as intermediate hosts for trematodes. For example, metacercariae of *Metagonimoides oregonensis* and *Diplostomulum desmognathi* infect certain aquatic desmognathine plethodontid salamanders (see Goater, 1990). These flukes use racoons and opossums, respectively, as their definitive hosts. Trematodes can also use herps as paratenic or transport hosts to bridge an ecological transmission barrier. Mesocercariae of *Alaria* spp., for

example, infect ranid frogs and colubrid snakes. Accumulation of mesocercariae in these more terrestrial hosts effectively bridges the aquatic-terrestrial barrier as the parasite relies on predation by canids such as foxes for transmission. An excellent review of the diversity of digenean trematode parasites (including keys) of amphibians is provided by Prudhoe and Bray (1982).

Reptile trematodes are primarily associated with hosts from aquatic habitats. Thus, freshwater turtles may be host to the lung flukes *Heronimus* spp. (e.g. Cox et al., 1988), as well as *Spirorchis* spp. which live in the circulatory system of marine and freshwater turtles (e.g. Ernst and Ernst, 1977; Esch et al., 1990; Platt, 1992; 2000). Semi-aquatic colubrid snakes also have a diversity of trematodes. For example, common flukes of garter snakes and water snakes include the lung trematode, *Pneumatophilus* spp., as well as several intestinal trematodes, including *Ochetosoma* (Rau and Gordon, 1980; Fontenot and Font, 1996). Keys to the Trematoda that will be valuable in identifying the flukes of reptiles can be found in Schell (1985).

Compared to the trematodes, the diversity of tapeworms (Class Cestoidea) in herps is low (Ulmer and James, 1976). Moreover, unlike adult trematodes that can live in a variety of tissues and organs, adult tapeworms are found only in the small intestine. Prudhoe and Bray (1982) should be consulted to help identify the tapeworms of amphibians. Common genera of amphibian cestodes are *Proteocephalus*, *Bothriocephalus* and *Cylindrotaenia* (e.g. Brooks, 1978; Jarroll, 1979; McAlpine, 1997; McAlpine and Burt, 1998). Reptilian cestodes include *Proteocephalus* spp. and *Ophiotaenia* spp. of freshwater turtles and semi-

aquatic colubrid snakes (Brooks, 1975; Ernst and Ernst, 1977; Rau and Gordon, 1980; Fontenot and Font, 1996)). Since cestodes rely on predator-prey pathways for transmission, several tapeworms have evolved life cycles that use certain amphibians and reptiles as intermediate (and/or paratenic) hosts and tertiary carnivores as definitive hosts, so tapeworm larvae can be found encysted in various tissues. For example, the tetrathyridia larvae of *Mesocestodes* spp. infect amphibians (e.g. McAllister and Conn, 1990; McAllister et al., 1995a, b), lizards (e.g. Goldberg and Bursey, 1991), and snakes (e.g. McAllister et al., 1991b; Bolette, 1997). The definitive hosts of *Mesocestoides* spp. are falconiform birds and various carnivorous mammals (Prudhoe and Bray, 1982). Werecommend the keys in Khalil et al. (1994) for identification of cestode parasites of reptiles

The worm parasites within the Phylum Acanthocephala (called thorny-headed worms, because of their recurved hooks on their protrusible proboscis) also use herps as intermediate and definitive hosts (McAlpine, 1996). Similar to the cestodes, adult acanthocephalans are exclusively parasites of the small intestine and all rely on predator-prey pathways in food webs for transmission. For example, adults of *Neoechinorhychus* spp. infect freshwater turtles (Ernst and Ernst, 1977, 1980; Aho et al., 1992) and use ostracod crustaceans as intermediate hosts (Esch et al., 1990). Examples of salamander acanthocephalans are *Pilum pilum* and *Fessisentis* spp. (e.g. Foard and Auth, 1990; McAlpine, 1996, 1997b). Several acanthocephalan species also use herps as paratenic hosts and larvae (cystacanths) encyst in the tissues. Cystacanths of *Pachysentis canicola*, for example, have been reported from the mesenteries of western diamondback

rattlesnakes (Bolette, 1997) and cystacanths of *Centrorhynchus conspectus* have been reported from the tissues of desmognathine salamanders (Goater et al., 1987).

Amphibians and reptiles are host to a diversity of roundworms in the Phylum Nematoda. Like the Trematoda, adult nematodes can live in a variety of tissues in the definitive host, although the small intestine is the most common habitat for roundworm parasites. Unlike the trematodes, many nematodes have evolved direct life cycles, although several orders of nematodes have complex life cycles with intermediate hosts. There appears to be little host specificity for the nematode parasites of herps, and differences in host behaviour and ecology (e.g. habitat and dietary preferences) are largely responsible for observed specificities (e.g. Goater et al., 1987; McAlpine, 1997a). In consequence, many common nematode species are found in a large range of hosts. Some of the most routinely encountered herp nematodes are in the genera *Capillaria*, *Rhabdias*, *Cosmocercoides*, *Oswaldocruzia* and *Falcaustra* (e.g. Baker, 1978; Baker et al., 1987; Coggins and Sajdak, 1982; McAlpine, 1997). Each of these genera consists of species found in a variety of herp orders, including reptiles. Other common reptilian nematode genera are *Spironoura* and *Camallanus* of freshwater turtles (e.g. Esch and Gibbons, 1967; Ernst and Ernst, 1977, 1980; Esch et al., 1990) and *Pharyngodon* and *Physaloptera* of lizards (e.g. Goldberg and Bursey, 1991; Hanley et al., 1995). Valuable comprehensive references dealing with the diversity and taxonomy of herp nematode parasites include Baker (1984; 1987) and Anderson (1992).

Pentastome Parasites

One of the most phylogenetically controversial and bizarre groups of vermiform endoparasites is the Pentastomida. Most pentastome species mature in the respiratory system of reptiles, especially tropical snakes and crocodilians (Almeida and Christoffersen, 1999). Most pentastomids appear to have complex life cycles involving intermediate hosts, e.g. mice in the rattlesnake pentastomid, *Porocephalus crotali*. Nymphs found in these rodent hosts, when eaten by rattlesnakes, penetrate the snake`s intestine and bore into the lung, where maturation occurs (Riley, 1981).

Leech Parasites

Three families of leeches, the Piscicolidae, the Hirudinidae and the Glossiphoniidae, contain representatives that are blood-feeding, usually temporary ectoparasites of aquatic vertebrates, including fish, amphibians and turtles. Two glossiphoniids of herp species include *Desserobdella picta* of various amphibians (e.g. Barta and Sawyer, 1990) and *Oligobdella biannulata* of desmognathine plethodontid salamanders (Goater, 2000). *Placobdella* spp. are primarily parasites of freshwater turtles (e.g. Light and Siddall, 1999). Several species of blood-feeding glossiphoniid leeches are vectors of haemogregarine and haemoflagellate protists that use amphibians and turtles as hosts (Siddall and Desser, 1991; Light and Siddall, 1999). Most leech species are temporary ectoparasites spending relatively short periods of time taking a blood meal from the vertebrate host. For

most of their life history they are free-living in the benthos. Thus, herp leeches may be a significant component of macroinvertebrate diversity in certain freshwater habitats. When found in high abundance in these habitats, glossiphoniids also serve as environmental stress indicators (e.g. Grantham and Hann, 1994).

Arthropod Parasites

Herps are also host to arachnid ectoparasites- ticks and mites. For example, larvae and nymphs of the tick, *Ixodes pacificus,* infect the fence lizard, *Sceloporus occidentalis*. This tick-lizard system is of medical significance because the tick is the vector of the Lyme disease spirochaete bacterium, *Borrelia burgdorferi* (Schall et al., 2000). Mites, including *Geckobiella* spp., also infect fence lizards and are vectors of the coccidian blood parasite, *Schellackia occidentalis*. Several tick species (e.g. *Amblyomma* spp.) have been recorded from terrestrial tortoises (e.g. Robbins et al., 1998). Ectoparasitic mites also infect some amphibians. For example, trombiculid mites in the genus *Hannemania* have been recovered from *Desmognathus brimleyorum* and various *Plethodon* spp. (e.g. Anthony et al., 1994; McAllister et al., 1995b). Larvae of this species (referred to as 'chiggers') invade amphibian tissue and become completely embedded in it (Anthony et al., 1994).

Certain highly specialized insects (Order Diptera, mostly within the families Sarcophagidae and Calliphoridae) also occasionally parasitize anuran amphibians. They are the causative agents of myiasis, typically killing their host after development of the larvae. A spectacular example of this form of parasitism is the sarcophagid fly, *Notochaeta bufonivora* that parasitizes harlequin frogs in Costa

Rica (Crump and Pounds, 1985). In this case, myiasis caused by *N. bufonivora* may be an important source of density-dependent mortality. Moreover, female frogs were more heavily parasitized than were males, and the parasite was hypothesized to be a cause of the heavily skewed sex ratio towards males (Crump and Pounds, 1985). Other larval dipterans have been reported infecting North American anurans. One example is the calliphorid fly, *Bufolucilia elongata* that parasitizes the American toad, *Bufo americanus* (Briggs, 1975). Species of Canadian anurans, including chorus frogs, wood frogs and boreal toads collected in Alberta, are parasitized by *Bufolucilia silvarum* (Brian Eaton, personal communication).

SAMPLING AND MONITORING OF AMPHIBIANS AND REPTILES

Many herps are secretive and inconspicuous and several are also fossorial and/or nocturnal. Moreover, at least for most amphibians, they have a complex, biphasic life cycle featuring an aquatic larval phase and a more terrestrial adult. As a consequence of these features, sampling techniques are quite different from other vertebrate groups. Considering the ecological importance of herps in many freshwater and terrestrial habitats, and given the declining status of many amphibian and reptile species, it is necessary to monitor populations accurately and, often, over the long-term (e.g. Pechmann et al., 1991; Skelly et al., 1999). Several different qualitative and quantitative sampling techniques have been designed to collect herps and/or monitor amphibian and reptile populations. Such quantitative monitoring techniques may not be of direct interest for those interested

in conducting a survey of the parasites of herps. Anyone who collects herps for a parasitological study should consider the comment of Heyer et al. (1994): “sampling procedures should be quantitative, so that an initial inventory can be extended into a monitoring program should the need arise”. This comprehensive reference reviews the standard methods for monitoring amphibians and can also be applied to many reptiles. For example, the standardized methodologies such as drift fences/pitfall traps and artificial cover objects, used for monitoring populations of terrestrial salamanders can be applied to certain snakes. Each of these, and all of the other sampling protocols, as well as topics such as field-based research design, techniques for marking amphibians, handling live amphibians and preparing amphibians as scientific specimens are all given comprehensive treatment in Heyer et al. (1994).

COLLECTION OF AMPHIBIANS AND REPTILES FOR PARASITOLOGICAL EXAMINATION

1. Collection of Herp Hosts

Herp collection protocols will depend on the questions to be addressed in the study. Ideally, specimens should be used in collaborative studies to maximize the information gained from each animal. In any case, there are some important issues to address before beginning any parasitological study. One issue is that of scientific collection permits. On ethical grounds and given the vulnerable status of many herp populations, this is important. In almost all cases, permits are required for destructive sampling of herps (or any vertebrate hosts for that matter). Before beginning any study, investigators should check with the appropriate agency for the

required scientific collection permits. All investigators should obtain the guidelines set out by the Canadian Council on Animal Care (web-site: http://www.ccac.ca/) for appropriate animal collection and ethical treatment protocols.

Parasite abundance and diversity in a given host reflect phylogenetic constraints, as well as geographic and host ecological and behavioural considerations. Moreover, parasite faunas can change dramatically as a function of time and space. For these reasons, locality and seasonal data are critical from a parasitological viewpoint. Thus, once the host has been correctly identified, the precise locality of the host and date of collection must be recorded. Most herps can be collected and placed in moist cloth bags, until returned to the laboratory and killed for parasitological examination. In rare cases, parasites may be sampled or monitored in the field without killing the host (e.g. Goater et al., 1990b; Wetzel and Esch, 1996a, 1997; Zelmer et al., 1999). Hosts collected from two ponds in the same geographic area can harbour very different parasites because of differences in food chain dynamics and/or intermediate host availability (e.g. Aho, 1990). Thus, herps from different populations, by definition, are collected from different localities and the labeling of field-collected herps must reflect this with accuracy and precision. Hosts should never be pooled across habitats. In addition, the transmission dynamics of parasites can vary significantly depending on time of collection. Thus, all hosts must be accompanied by an accurate label indicating the date of collection.

The number of hosts collected depends on the question to be addressed. However, for most purposes and for future statistical analyses, a sample size of approximately 20 individuals within a given class (e.g. age, size, sex, locality, month) is recommended. Obviously, the more hosts examined, the greater the possibility of encountering rare parasites. It should also be noted that fresh, intact road-killed herps can provide valuable sources for parasitological studies,

especially rare or endangered herps that cannot be destructively sampled. However, if the investigator is interested in ecological questions, the dead herp must be accompanied with the appropriate habitat and date of collection data as described above. Herp hosts have also been analyzed for parasite diversity by taking advantage of large numbers of hosts deposited in certain vertebrate museums (e.g. Goldberg and Bursey, 1991; Goldberg et al., 1998).

Whenever possible, herps should be collected across their natural size range. Most herps undergo pronounced ontogenetic niche shifts as they grow (e.g. Werner, 1986) and many herps exhibit pronounced sexual dimorphism (e.g. Howard, 1981; Shine, 1994). Thus, age-related and/or sexual differences in body size may alter food web relationships, and the nature of predator-prey, as well as host-parasite interactions (e.g. McAlpine, 1997). Consequently, the herp's SVL and weight must be recorded on the necropsy data sheet (see OVERVIEW OF NECROPSY PROCEDURES section).

2. Methods of Euthanasia

Humane treatment of the herp should be the goal of all concerned with the necessity of killing the host for endoparasitic examinations. The correct method of euthanasia will vary with the taxa of herp in question. For example, exposure to a concentrated dose of an anaesthetic, such as tricaine methane sulfonate (MS-222), or chlorotone, or sodium pentobarbital (Nembutal), is the preferred method for salamanders, frogs and turtles (e.g. Goater et al., 1987; Muzzall, 1991b; McAllister et al., 1995a, b). Pithing is also a recognized method of sacrificing amphibians, especially anurans (e.g. Joy and Pennington, 1998). Snakesare best killed by intracardial injection of MS-222 (e.g. Fontenot and Font, 1996) or sodium

pentobarbital (e.g. Platt, 2000). Snakes and lizards can also be sacrificed by cold narcosis and decapitation (e.g. Hanley et al., 1995).

3. Further Host Handling and Data Collection

Preferably, herps should be examined fresh for endoparasites, within 24 hours, whenever possible. This can be important for a variety of reasons depending on the types of parasites to be encountered and the questions of interest. First, live parasites are more easily and quickly observed in tissues and intestinal debris, as well as blood smears and fecal samples. Second, live parasites that are then adequately fixed and preserved provide the best material for future identification purposes.

It is rarely practical to immediately examine a large sample of freshly killed hosts for parasites. Alternatively, hosts should be frozen as soon as is possible after capture. This can be done efficiently in the field using either an ethanol and a dry ice mixture (e.g. Bush and Holmes, 1986) or liquid nitrogen, or immediately upon return to the laboratory. If using the latter, we prefer quick-freezing in an ultra-cold freezer, as this limits post-mortem migration of parasites. We recommend, recognizing potential time constraints, that careful analysis of host stomach contents be part of the parasitological examination; immediate freezing increases the value of stomach content analyses since digestive processes are retarded and prey contents will not be biased. An organism's feeding habits are an important feature of its natural history. Furthermore, diet plays a crucial role in the transmission of many helminth parasites with complex life cycles. Greater insight into the biology of a herp as well as information on the potential transmission of the parasites recovered is gained by including stomach content analyses. . Finally, the integration of stomach contents and parasites will help in understanding the nature

of the predator-prey interactions in the sampled habitat(s). Quick freezing of hosts may also be valuable at a later date if anyone is interested in obtaining specimens for molecular or phylogenetic studies. Excellent museum quality parasite and host specimens can be prepared from herps that have been carefully collected, labeled and quick-frozen. Voucher specimens representing the hosts should be archived in the event that there are systematic problems with the hosts later (especially if there are current unresolved systematic issues with hosts). Obtaining data in addition to the parasites present clearly increases the time required to necropsy each host, but in many cases the extra time may be justified, particularly for herps which are difficult to collect. In such cases, future samples of hosts may simply not be available and researchers should aim to collect as much information as possible.

All hosts should be bagged separately and all pertinent information recorded on labels to be placed in the bag prior to freezing. Data that should be included with each individual host include the species, precise locality and date of collection (see above) as well as the collector. These will be placed on a data sheet at the time of necropsy. A sample data sheet for the parasites of freshwater fish prepared for EMAN by David Marcogliese (web-site: <A HREF="http://eqb-dqe.cciw.ca/eman/ecotools/protocols/freshwater/parasites/appendix1.html">) can be slightly modified for herps.

As mentioned earlier, prior to examination of a host for parasites, the following other host information should be recorded on the necropsy data sheet: sex, snout-vent length (SVL) and weight. For many herps, correct identification of sex will require dissection of the animal to examine reproductive organs.

EXAMINATION OF HERPS FOR PARASITES: OVERVIEW OF NECROPSY PROCEDURES

1. Literature Search

It should be recognized that an overview of standardized protocols for parasitological examination will not apply completely to every herp taxon. The following is a framework we have found useful in dealing with the parasites of herps. These parasitological protocols for examination of reptiles and amphibians were modified from Prudhoe and Bray (1982) and Doster and Goater (1997). As described earlier, helminths and protists can be found in almost any organ, tissue or cavity of a herp's body. Thus, it is impractical to provide an exhaustive or precise set of protocols for all parasites, so we have provided references to the relevant literature to guide the reader to more specific procedural information.

The "expected" parasite fauna is an important consideration in planning appropriate survey methods for various herp species (see Doster and Goater, 1997). Before beginning any parasitological study one should first ascertain what parasites have been reported from a particular host, or related hosts, preferably in the same geographic area as the proposed study. A close reading of the numerous references (and the references therein) specific to each parasite taxon we included earlier (see Types of herp parasites: an overview) is an important first step in obtaining the necessary literature for the herp(s) in question.

2. Equipment

The equipment required to search for herp parasites includes high quality dissecting equipment such as scissors, scalpels, fine forceps and dissecting needles. Other essential necropsy equipment includes squeeze bottles with water, the various parasite preservatives, stains and clearing agents (see below), Pasteur pipettes, Petri dishes, vials for specimen storage, slides and coverslips. The necropsy station will also have a high quality compound microscope (for examination of protists and for species identification of macroparasites) and a dissecting microscope (for examination of host surfaces and internal organs for macroparasites). Some additional equipment is required for specialized procedures, depending upon the goals and expertise of the investigator (see relevant sections and references below).

3. Overview of Necropsy Procedures: Macroparasites

a. Examine the external surface with the naked eye and, if appropriate, under the dissecting microscope for leeches and ectoparasitic ticks or mites.

b. Examine buccal cavity visually and then under the dissecting microscope.

c. Examine eyes and thin-slice musculature for encysted parasites, especially in aquatic hosts for metacercariae of digeneans (e.g. Marcogliese et al., 2000).

d. Use scissors to slit the ventral surface and open the body cavity from mouth to cloaca, record sex.

e. Visually examine the body cavity, mesenteries and external surface of internal organs, repeat with dissecting microscope.

f. Separate all internal organs into Petri dishes with water.

g. Separate digestive system into esophagus, stomach, intestine and colon sections and place separately into Petri dishes with water. Open each section longitudinally and examine digestive debris for adult helminth parasites with dissecting microscope.

h. Cut internal organs and carefully tease apart tissues into smaller pieces, then examine for larval and adult helminth parasites with dissecting microscope. Collection of tissue samples and fixation in buffered formalin from the various internal organs is also encouraged. Collection of such samples enables subsequent preparation of wax blocks and histological slides for examination of protists and myxozoans (John Barta, personal communication).

i. Record number of parasites of each species and their location in the host on the necropsy data sheet (see sample data sheet at the following web-site: <A HREF="http://eqb-dqe.cciw.ca/eman/ecotools/protocols/freshwater/parasites/appendix1.html">)

j. All recovered macroparasites should be fixed and preserved in labeled vials using conventional parasitological techniques. There are many tricks and suggested protocols that are specific to each macroparasite taxon. Investigators must adopt the conventional protocols for handling macroparasite material so that accurate identification can be made by experts, and so that parasites can be deposited in museums for future comparison purposes. Fixation procedures and staining protocols for permanent slide making of platyhelminths, the

procedures for making temporary mounts (e.g. nematodes), and the types of preservatives (and recipes) can be found in any parasitological lab manual (e.g. Ash and Orihel, 1991). Many of the references we have listed under the TYPES OF HERP PARASITES: AN OVERVIEW section contain protocols specific to herp parasite diversity studies and these should also be consulted. Great care should be taken to save all parasites for future identification. For example, it should be recognized that two closely related species may live together or very near each other in the same host. It should never be assumed that all the parasites from a given region in the same host are the same species. Only by careful collection of parasite specimens and meticulous attention to labeling protocols will later confusion and inaccuracies be avoided. In some dioecious parasites (e.g. nematodes) males and females also look very different at necropsy and may be recorded as different species. This will not matter if all parasites are collected, fixed, preserved and labeled accurately for precise enumeration and identification at a later date. In general, live platyhelminths and leeches must be killed and fixed so that they are as elongated and dorsoventrally flattened as possible. Worms can be placed in saline or warm water for relaxation. Often worms will release eggs which is advantageous because an egg-filled uterus can hide the important reproductive structures which are essential for identification. Alternatively, live worms can be placed hot 5% formalin for relaxation and elongation and then post-fixed in AFA. This fixative, a mixture of 4% formalin, 70% alcohol and 10% acetic acid, is widely used for fixation of platyhelminths. Placing the fluke or tapeworm to a slide and applying very gentle coverglass pressure

while bathing with AFA can help ensure killing and flattening of delicate worms for identification. Great care must be taken not to distort the internal organs of the worms if this technique is used. The detailed structure of reproductive organ systems of platyhelminths often requires specimens to be studied in serial sections (e.g. Zelmer and Brooks, 2000) and improperly flattened specimens are often useless for this purpose. Correctly fixed worms can then be stored in labeled glass vials containing 70% alcohol. The same protocols can be applied to acanthocephalans; relaxation of these worms is important to ensure protrusion of the proboscis, a valuable taxonomic feature. Nematodes can be fixed in 5% formalin and then placed in vials with a solution of 5% glycerin and 70% alcohol, and arthropods can be placed directly into vials containing 70-80% alcohol. Preserved parasites can be held in these vials indefinitely.

k. In brief, platyhelminths and leeches are examined microscopically as whole mounts after the specimens have been stained with a haemotoxylin or a carmine stain (e.g. Meyer's haemotoxylin or Semichon's acetocarmine) and then dehydrated by putting them through an ascending graded series of alcohols. Specimens are then cleared in a clearing agent (e.g. xylene, cedarwood oil, clove oil) and mounted on microscope slides in a mounting medium such as Canada Balsam (NOT Permount). Canada Balsam is the conventional high quality archival mounting media suitable for making voucher specimens. Nematodes are best studied and identified in temporary whole mounts (stains do not penetrate the cuticle) using the clearing agents lactophenol or glycerol. Arthropods can be studied and identified in temporary mounts or cleared and mounted on slides for

microscopic examination and preparation of voucher specimens. Laboratory suppliers of the reagents used in parasitological studies can be found in the table prepared by David Marcogliese (see web-site at <A HREF="http://eqb-dqe.cciw.ca/eman/ecotools/protocols/freshwater/parasites/table2.html" >

3. Overview of Necropsy Procedures: Microparasites

Some microparasites can be examined by following the procedures for macroparasites (e.g. large intestinal protists). However, most require complex, specialized protocols, depending on the tissues or organs of residence. Only a general methodology is provided; investigators should consult the references listed for specific details. Generally, most parasitic protists of herps are found in the blood-stream, within organs, or in the digestive system.

Blood smears for the detection of trypanosomes and intracellular coccidians can be made by snipping the tail or toe of the herp in the field (or a freshly killed host) and spreading the blood evenly against a microscope slide. Toe snips or tail clips can produce very watery blood. Therefore, vascular blood (either obtained by by puncture or post-mortem is usually better for parasite identification of blood forms (John Barta, personal communication). After smearing, the blood is allowed to air dry, fixed in 10% buffered formalin or methanol and stained in Giemsa or Wright's stain, then stored for future examination (e.g. Jones and Woo, 1991). It is often useful to use air-dried smears that are fixed in absolute methanol and then air-dried again before staining; this has the advantage that the slides can be air-dried and methanol-fixed in the field and then returned to the lab for staining

and long-term storage (John Barta, personal communication). Alternatively, and preferably, the hematocrit centrifuge technique pioneered by Woo (1969b) can be used. This involves collection of blood (cardiac or caudal vein blood) into heparinized capillary tubes, centrifugation in a microcapillary haematocrit centrifuge and then examination under a compound microscope at 100X magnification (e.g. Woo and Bogart, 1986). Microscopic examination of stained kidney impressions have also been used for the detection of trypanosomes of amphibians (Jone and Woo, 1989). Woo (1983) and Godfrey et al. (1987) should be consulted for further details regarding the limitations of dealing with quantification of haematozoan parasites. Fecal samples can be examined for the presence of extracellular coccidian parasites.

Coccidian oocysts can be concentrated from fecal samples by flotation in an aqueous Sheather's sucrose solution and monitored under phase contrast optics of a compound microcroscope (e.g McAllister and Upton, 1987; Hanley et al., 1995). Specific details for coccidial isolation from herps can be found in McAllister et al. (1995a). Protist and myxozoan parasites within organs can be studied within representative tissues mounted in wax blocks that are then sectioned. Tissue sections are then stained and mounted on slides for examination of microparasites.

4. Quantification of Parasites of Herps

For many herp-parasite associations, methods for quantifying the number of parasites within a host are a straightforward extension of the methods used to collect parasites for identification. Herps are collected and preserved (as described earlier), and their parasites are isolated from specific organs and counted under a dissecting microscope. This traditional "kill, cut and count" approach can be used to accurately quantify many of the macroparasites. For other parasites, such as the

haematozoans and coccidians, quantification is extremely difficult, often unreliable and inaccurate, and other methods are required.

The two critical quantification measures are **prevalence** (the percentage of hosts infected with a given parasite) and **intensity** (the number of parasites of a particular parasite in infected hosts (see Margolis et al., 1982; Bush et al., 1997 for a thorough discussion of these and other ecological terms in parasitology). Mean intensity, for example, is a common measure used in ecological parasitology and refers to the mean number of parasites of a given species per infected host. Prevalence and intensity data for macroparasites can be obtained using the “kill, cut and count approach” because individual parasites can be relatively easily isolated, the immature stages are typically macroscopic and, for herps, rarely exceed intensities of approximately 100 within an individual host. In such cases, intensity can represent a fully-censused parasite population. The reliability of counts of large helminths in hollow organs (e.g. intestine) may be substantially higher than those made on helminths from tissues. For the latter, intensity comes from counts made on helminths which have been 'teased-out' under a dissecting microscope. Smaller species or immature stages (or males of sexually dimorphic species, such as nematodes) can easily be missed. One way to address this problem is to squash samples of tissue (which have been teased apart) between two glass plates and examine the preparation under a dissecting microscope. In the process of collecting intensity data, other important characteristics of helminth populations can be considered, depending on the aims of the study. One characteristic that is seldom considered is total worm biomass/species. This can be measured as total wet or dry weight, from which estimates of mean *per capita* worm weight can be determined (e.g. Goater, 1992). Mean worm size (measured as length or volume) may also be a useful population characteristic. An indication

of the reproductive status of individual worms (e.g. counts of the number of eggs in the uterus) may also be valuable (e.g. Joy and Pennington, 1998).

The quality of intensity data obtained from hosts collected for the specific purpose of helminth quantification can be expected to be higher than for herps collected for other reasons (e.g. found dead). Differences in data quality may be especially obvious when features such as worm weight, size, and reproductive status are of interest. The use of road killed herps, for example, involves hosts that are killed at different times, preserved (usually frozen) at different times, and collected under different environmental conditions. Our experience is that the use of such hosts may provide reliable intensity data for certain parasites (e.g. large nematodes), but may provide less reliable information for the small, soft-bodied platyhelminths.

Compared to most species of helminths, quantifying protist intensity is extremely difficult. Although there are numerous techniques available for the clinical diagnosis of protist infections, and these can provide prevalence data, there are few examples of attempts to determine intensity. The main problem is the high variability in parasite counts, even in tightly controlled laboratory systems. Much of this variability is a characteristic of the life-histories of protist parasites, and is particularly due to inherent changes in intensity during the infection period. However, variability can also reflect the specific methods used, and the experience of the investigator. Despite the problems associated with such high inherent variability, and its interpretation, it is important to provide some indication of intensity for protists. Each of the common herp parasite genera can be isolated and quantified using the techniques for preparing and examining blood films or fecal smears that were described above. Intensity is generally estimated by examining stained blood films under a compound microscope. For example, in the case of haematozoans, the overall goal is to obtain an intensity estimate based on

the number of parasites (or numbers of infected erythrocytes) per number of uninfected erythrocytes, or counts in a fixed number of microscope fields or a fixed period of time. Godfrey et al. (1987) outline certain limitations of these techniques which have been used to determine intensity of haematozoans in birds and make several recommendations for improving and standardizing methods.

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QUALITY CONTROL/VOLUNTEER SUPPORT

A quality control/assessment plan is required for any successful monitoring program. It gives results credibility and helps structure and organize the monitoring program. There are several ways to structure a herp-parasite program. These include keeping comprehensive field notes, maintaining strict and precise sample collection protocols of both hosts and parasites, identifying the procedures for verifying taxonomic identifications, identifying the methods of data analysis, and maintaining sound database management. Questionable or uncertain parasite

identifications should be verified by an expert (see EXPERTS TO CONTACT section).

People do not require a great deal of training to do adequate dissections to recover internal helminth parasites. However, they do require initial guidance by an expert to make sure they know what they are looking for and understand the techniques required. Once these have been mastered, volunteer undergraduate students, for example, can acquire a lot of valuable data. However, people with some expertise or experience are required for making the correct identifications. Databases collected by volunteer groups should be maintained independently from those of experts until verification of species is assured.

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EXPERTS TO CONTACT

There are several Canadian scientists with extensive research experience dealing with amphibians and reptiles and their parasites. Some of these scholars, their mailing addresses, e-mails and their expertise are listed here. Stephen Upton is also included because of his expertise with coccidian and myxozoan parasites of herps.

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LINKS TO PARASITOLOGICAL/HERPETOLOGICAL INTERNET RESOURCES

http://www-museum.unl.edu/asp/ The American Society of Parasitologists web-site; the definitive site for North American parasitologists, with numerous links to parasitological resources.

http://www.biology.ualberta.ca/parasites/home.htm The Canadian Society of Zoologists-Parasitology section web-site, providing a directory of Canadian parasitologists.

http://eqb-dqe.cciw.ca/emanops/intro.html The Ecological Monitoring and Assessment Network (EMAN) web-site maintained by Environment Canada. Specifically, this link accesses the EMAN observational programs and the valuable new FROGWATCH program designed for Canadians to study amphibian natural history and to monitor amphibian populations in each province. Many valuable amphibian links can be found here.

http://eqb-dqe.cciw.ca/partners/carcnet/ The Canadian Amphibian and Reptile Conservation Network (CARCNET) web-site, with detailed information on the natural history and conservation status of Canadian herps.

http://www.on.ec.gc.ca/herptox/ A web-site from Environment Canada that examines the role of environmental contaminants and toxicology on reptiles and amphibans

http://www.mp1-pwrc.usgs.gov/amphibs.html The North American Amphibian Monitoring Program web-site, concerned with the monitoring and conservation of amphibians.

http://www.ukans.edu/~ssar/SSAR.html The Society for the Study of Amphibians and Reptiles web-site, with links to many herpetological resources. Publishers of "Journal of Herpetology".

http://www.utexas.edu/depts/asih/ The Society of Ichthyologists and Herpetologists web-site, with links to many herpetological resources. Publishers of the journal "Copeia".

http://www.cdc.gov/ncidod/eid/vol5no6/daszak.htm . An excellent peer-reviewed review paper entitled "Emerging infectious diseases and amphibian population declines" by Peter Daszak and colleagues, published in "Emerging Infectious Diseases" by the National Center for Infectious Diseases in 1999 (Volume 5).