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A Practical Guide for the Study of Malformed Amphibians and Their Causes

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ABSTRACT.—**Reports of severely malformed amphibians in the 1990s prompted researchers to examine the causes and extent of the issue. However, disparities in survey methods and a shortage of baseline data have hindered standardization among investigations. Here, we review field-based surveys and experimental approaches used over the last decade to address this complex ecological issue. We offer specific recommendations regarding amphibian-sampling techniques, including methods to capture and examine amphibians, with the aim of enhancing the accessibility of this topic to scientists, students, and educators of diverse backgrounds. Based on established approaches from epidemiology, we provide recommendations regarding methods to identify proximate causes(s) of abnormalities with a focus on using "multiple lines of evidence," including large-scale field surveys, comparing malformation "signatures" between field and laboratory studies, and using of manipulative experiments at multiple spatial scales. As an example, we describe methods to examine the causal influence of infection by the trematode parasite, *Ribeiroia ondatrae*, including quantifying presence and abundance within amphibian and snail host populations with adequate power of detection. We conclude by identifying outstanding questions with the goal of stimulating additional research to evaluate the causes and consequences of amphibian malformations.**

In August 1958, Royal Bruce Brunson, a biologist at Montana State University, made an alarming discovery: a pond with large numbers of severely deformed Pacific Chorus Frogs (now known as Pacific Treefrog; *Pseudacris regilla*) in Montana. Approximately 20% of emerging frogs exhibited severely malformed limbs, including extra limbs, missing limbs, and a variety of twisted and otherwise misshapen limbs (Hebard and Brunson, 1963). Brunson again noted high frequencies of malformed Chorus Frogs at this pond in 1959, 1960, 1961, and 1964 (R. Brunson 1999, pers. comm.), whereas others found deformities at the same site through the 1970s and into the 1990s (Miller, 1975; Anderson, 1977; J. Werner, pers. comm.). Given the rise of nuclear power, some feared such deformities were caused by radiation, whereas others suggested pesticides used for mosquito control. However, no evidence was available to support these hypotheses. Investigators generally assumed that, although remarkable, the phenomenon was an isolated or at least a very rare occurrence (e.g., Hebard and Brunson, 1963), such that real cause would remain unknown for many years to come.

Four decades later, discoveries of multiple wetlands across the United States with large numbers of malformed amphibians demonstrated that this phenomenon was neither isolated nor rare (Sessions and Ruth, 1990; Helgen et al., 1998; Johnson et al., 1999; Kaiser, 1999a,b; Converse et al., 2000; Hoppe, 2000; Souder, 2000; Johnson et al., 2002, 2003; Kiesecker, 2002; Eaton-Poole et al., 2003; Lannoo et al., 2003; McCallum and Trauth, 2003; Vandenlangenberg et al., 2003; Hoppe, 2005; NARCAM, 2010). These reports often documented populations in which greater than 10% and even up to 95% of amphibians suffered from severe limb malformations. Malformations primarily affected the limbs of recently emerged individuals, typically from lentic habitats such as ponds and lakes. Particularly troubling was evidence from museum-based and historical studies suggesting that, over the last several decades, malformations in amphibians have become more widespread, more severe, and often affect a higher proportion of individuals in a population than observed previously (Gray, 2000; Hoppe, 2000; Johnson et al., 2003; Johnson and Lunde, 2005).

Research conducted by academic groups as well as local, state, and federal agencies has identified important causes of amphibian malformations. Although dozens of agents can potentially induce amphibian malformations in laboratory settings (reviewed by Ouellet, 2000; Stopper et al., 2002), four factors emerged as the most likely candidates to explain contemporary observations of limb abnormalities in wild populations: (1) pesticides; (2) UV-B radiation; (3) injury from predators; and (4) parasites (see reviews by Blaustein and Johnson, 2003; Sessions, 2003; Ankley et al., 2004; Johnson et al., 2010). Substantial progress has been made in understanding the role of these four causes in causing limb malformations in North American amphibians. Research conducted by the EPA, for example, argued against UV-B as a cause of deformities because elevated exposure resulted in bilaterally symmetric malformations, which are rare in natural populations (Ankley et al., 2004). Research on contaminants such as pesticides has suggested a linkage between abnormality levels and land use (e.g., Ouellet et al., 1997; Kiesecker, 2002; Taylor et al., 2005; Reeves et al., 2008, 2010), yet no particular pesticide or chemical has been identified as a direct cause. Efforts are underway to identify potential compounds using field and laboratory experiments (e.g., Bridges et al., 2004; McDaniel et al., 2004; Loman and Lardner, 2006; Brunelli et al., 2009; Spolyarich et al., 2010). A series of recent studies have further revealed that aquatic predators can cause amphibian limb abnormalities, including missing and shortened limb abnormalities in Oregon, the Northeast, and Alaska (Ballengée and Sessions, 2009; Bowerman et al., 2010; Reeves et al., 2010).

One of the most well-studied recent causes of amphibian malformations is infection by the digenetic trematode, *Ribeiroia ondatrae*. Experimental exposures involving field-based levels of *Ribeiroia* infection cause skin webbings, bony triangles, partially and completely missing limbs, extra limbs, and otherwise abnormal limbs in frogs, toads, and salamanders (Johnson et al., 1999, 2001, 2006, 2008, 2012; Sessions et al., 1999; Kiesecker, 2002, Stopper et al., 2002; Schotthoefer et al., 2003). *Ribeiroia* infection has been linked to both contemporary and historical accounts of "mass malformations" in naturally occurring amphibian populations, including Dr. Brunson's long-enigmatic Jette Pond in Montana (Johnson et al., 2002, 2003, 2006; Kiesecker, 2002; Lannoo et al., 2003; Sutherland, 2005; Johnson

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and Hartson, 2009). However, infection by this parasite cannot explain all types of malformations, nor is it present at all sites with large numbers of malformed amphibians. For example, *Ribeiroia* was not present at several sites with high levels of frogs with missing legs in Minnesota (Lannoo et al., 2003; Lannoo, 2008) nor at any sites with malformations in Alaska (Reeves et al., 2008; Reeves et al., 2010). In addition to *Ribeiroia*, other parasites have been suggested or shown to cause morphological abnormalities in amphibians, including monogeneans (Rajakaruna et al., 2008), parasitic arthropods (Murphy, 1965; Kupferberg et al., 2009), and viruses (Ouellet, 2000).

Although our understanding of the amphibian malformation issue has progressed considerably over the past decade, a number of factors still hinder scientific advancement. First, field sampling methods differ substantially among research groups, often preventing standardized comparisons across studies. Second, there have been few methodologically oriented papers that discuss approaches to sampling amphibians, and no paper to date has attempted to evaluate what lessons can be learned from various approaches or recommend overall guidelines for conducting large-scale malformation surveys. Third, field surveys have suffered from a lack of information regarding the expected baseline levels for malformation prevalence, limiting the ability of researchers to classify and identify which wetlands or regions are exhibiting unusual levels of abnormalities. Finally, there has been little discussion regarding what data are necessary to determine the causative agent(s) at sites with a large percentage of malformed amphibians or how to collect and analyze such data.

The purpose of this article is to review available information on field-based malformation surveys and analysis methods with the goal of providing suggestions for developing efficient and consistent protocols. Our intended audience includes academic researchers, local and federal agency personnel, and educational groups or classes across a wide range of student ages. In particular, we describe the statistical tools necessary to classify sites with a higher than expected prevalence of abnormalities and discuss issues regarding sample size and site selection. We further discuss what data are required to evaluate the influence of potential causes by drawing upon example models from disease ecology and epidemiology. Finally, we outline field and laboratory protocols to examine populations of amphibians and snails for *Ribeiroia* parasites. It is our hope that such information will allow researchers to better communicate with each other, facilitate broad scale meta-analyses, and investigate the issue in a rigorous and scientifically defensible manner.

Practical Guide for Field Surveys of Malformed Amphibians

Two major goals of this section are to make the study of amphibian malformations more accessible to scientists of diverse backgrounds and to develop more consistent methods by offering recommendations on how to sample amphibians and analyze the resulting data. To this end, we have synthesized information on field protocols and statistical techniques to establish informed methodologies for studying malformations and encourage new research to answer many pressing questions. Fundamental to this discussion is consistent use of terminology, which is based on Meteyer (2000), Johnson et al. (2001b), and USFWS (2008). In this paper, we use the term "abnormality" to refer to any deviation from normal morphology, independent of whether its origin was developmental or acquired after proper development (i.e., injury). We use "malformation" to refer to a deviation from normal morphology resulting from improper development, which might be attributable to teratogens, genetic anomalies, or developmental errors. Where appropriate, we also refer the reader to other sources with more detailed information (e.g., the USFWS Amphibian Abnormality Protocol, http://training.fws.gov/EC/frog/frogabnormalitytrainingmanual.pdf).

Amphibian populations with a high prevalence of limb abnormalities have been reported in regions beyond North America historically and currently, including Eurasia (Rostand, 1949; Woitkewitsch, 1959; Dubois, 1979; Henle, 1981; Borkin and Pikulik, 1986; Veith and Viertel, 1993; Van Gelder and Strijbosch, 1995, Bohl, 1997), India (Gurushankara et al., 2007), Japan (Meyer-Rochow and Asashima, 1988), Bermuda (Bacon et al., 2006), and Australia (Tyler 1998; Spolyarich et al., 2011). Although the methods, taxa, and wetland types discussed in this paper are designed to be most applicable for surveys in North America, the lessons and study goals apply to researchers in temperate and tropical regions.

How to Select Study Sites.—Most malformation reports involve amphibian species with larvae that develop in ponds, streams, or lakes (Johnson et al., 2010). Wetlands reported to support the largest populations of malformed frogs tend to be small, lentic (still-water) habitats that are either anthropogenic in origin or altered by human activity. For example, cattle ponds and farm ponds are common malformation hotspots in the Western and Midwestern United States, respectively (Johnson et al., 2002; Lannoo et al., 2003). As of 1999, amphibian abnormalities had been documented in 44 states (Kaiser, 1999b), suggesting field surveys are warranted in any region, state, or locality. Moreover, amphibian abnormalities have been documented in 71 species, and the most commonly affected groups are frogs and toads (Lannoo, 2008).

Because of the diversity of sites and species involved, the sampling design used to include sites (e.g., wetlands, streams, ponds) in a malformation survey can influence what conclusions may be derived from the data. The majority of initial malformed frog surveys targeted wetlands with recent or historical reports of malformations (Sessions and Ruth, 1990; Gardiner and Hoppe, 1999; Johnson et al., 2002). Such targeted designs are likely to encounter a large number of sites with high levels of malformations and, thus, are useful to identify agents causing malformations, to determine which amphibian species are most susceptible to malformations and which malformation types are most common, or to examine the effects of malformations on amphibian population dynamics. Surveys targeting malformation sites can also incorporate a case-control design by pairing targeted sites to nearby wetlands with no known history of malformations (e.g., Johnson et al., 2002; Vandenlangenberg et al., 2003). Such a survey design allows for a comparison between malformation prevalence and levels of suspected causative agents between the two types of sites. However, researchers must exercise caution in how the data are extrapolated given that site-selection is nonrandom and cannot be extended to other types of habitats or regions not under study. For instance, in the largest study of amphibian abnormalities to date conducted by the U.S. Fish and Wildlife Service (USFWS, unpubl. data), researchers have sampled frogs from National Wildlife Refuges across the United States. This provides an excellent picture of abnormality patterns from such refuges, but the study design cannot be used to extrapolate to nonrefuge habitats (e.g., wetlands on private properties or farms).

A third type of survey design involves using a probabilistic design, in which sample sites are drawn from a larger, previously identified pool of suitable sites has been applied by Environmental Protection Agency and many state biomonitoring programs (Olsen and Peck, 2008). This design, which has rarely been applied to the study of frog abnormalities, can be used to determine associations between malformations and factors such as ecoregion or land use (e.g., Schoff et al., 2003; Reeves et al., 2008) and has the advantage of providing a more representative picture of the occurrence of malformations across a landscape. This same survey design can also be used to provide baseline data for future surveys to determine whether malformation levels are increasing over time. One weakness of a probabilistic design is that some selected wetlands may not support amphibians, and a large number of wetlands must be surveyed if only a small proportion are expected to have significant numbers of amphibian with malformations.

Which Species and Life Stage to Examine.—Whether malformations are detected at a given wetland can be influenced strongly by what amphibian life stage and species are sampled. Abnormalities are most common in late stage larval and recently metamorphosed amphibians but nearly absent in adult populations (Johnson et al., 2010). It has been suggested that this difference results from reduced survivorship among the malformed metamorphic frogs, possibly from increased predation or inability to capture prey (Goodman and Johnson, 2011). Therefore, among anurans (frogs and toads), targeted sampling at the metamorphic stage (just prior to or recently after metamorphosis) is optimal because (1) it allows researchers to nonlethally inspect large sample sizes in the field and (2) all skeletal features have developed by metamorphosis, such that any external malformations will be visible. In contrast, inspection of abnormalities among anuran larvae is most reliably performed with a stereo dissection microscope, which requires either anesthetizing or euthanizing large numbers of individuals for transportation to a laboratory facility. Although field inspections of late-stage larvae (i.e., after Gosner [1960] stage 42) may be feasible for large species, this approach is likely to underreport small and early stage abnormalities among small anurans. Furthermore, lab-held animals generally should not be returned to field sites without careful examination or treatment for pathogens potentially acquired in captivity. If the target species is a salamander or newt, sampling near-metamorphic individuals is optimal because the limbs and digits have fully developed and capture success tends to be high. In general, adult amphibians are less informative for malformation surveys because (1) they rarely exhibit malformations above baseline levels, even when 50% of the cohort's metamorphic frogs are malformed (Lunde et al., 2012); (2) the present location of an adult may not represent their natal pond or stream; and (3) collection of this life stage is likely to have the greatest impact on the amphibian populations.

Although field surveys may be designed to sample multiple species of amphibians per wetland, to facilitate comparisons among study sites and geographic regions, researchers can also use a "focal amphibian species" or genus that is likely to be present across the range of a given monitoring program and is known to be affected with malformations. The focal species should be common, have a large geographic range, and be abundant when present. In the Western United States, the Pacific Chorus Frog (*Pseudacris regilla*) fits these criteria (Rorabaugh and Lannoo, 2005). In addition, owing to their small size, *P. regilla* are easy to catch by hand, regardless of abnormality status, which limits sampling bias. In the Midwestern and Northeastern United States, the Northern Leopard Frog (*Lithobates pipiens*) is a potential focal species in light of its large geographic range, which includes a third of the United States as well as southern Canada (Rorabaugh, 2005). Monitoring of these focal taxa when present allows researchers to more easily make statistical comparisons of malformation prevalence among sites and among studies. It is important to stress, however, that the suggestion to include a focal species is not an argument to study only these species. Certainly, studies are needed to document malformations among rarer species and those in decline provided such studies do not adversely affect their populations.

How and When to Sample for Amphibian Malformations.—The best methods for capturing amphibians depend on the species and life-history stage of interest. Larval amphibians can be sampled using active sweeps with a dip net as documented in sampling guides (Heyer et al., 1994; Olson et al., 1997; Dodd, 2010). For metamorphic anurans, we recommend using species-specific, fixed time-transects, while recording the area covered and the estimated percentage capture efficiency to allow for density estimates and comparisons among observers. Toad metamorphs are often easily captured by hand while searching around the wetland shoreline or among cracks in the drying soil. Hylid and ranid frog metamorphs can be captured by hand or with a dip net along the shoreline. Although most species can be sampled during the day, some may be easier to capture at night with the aid of a headlamp. Animals should be handled with care to avoid causing injury or spreading pathogens (see below).

During each transect, captured animals of the same species can be stored in a moistened Ziploc bag, plastic bucket, or pillowcase. Care should be taken to ensure that animals do not become overcrowded or overheated, which will depend on the species being collected, the container size, and ambient temperatures. For small wetlands, all transects can be completed prior to inspections such that no animal is sampled twice. Recapture of the same individual is unlikely in larger wetlands (>5 ha) where transects areas can be distributed along the shoreline. If infection by chytrid fungus (*Batrachochytrium dendrobatidis*) or another pathogen is of concern or if the targeted species is threatened, each individual should be inspected immediately using a clean pair of nitrile gloves to reduce the potential of disease transfer or handling stress. To prevent the spread of disease and unintentional species introductions, all sampling equipment (nets, trays, waders) must be rigorously sterilized between sampling sites following established protocols, such as a 15-min soak in 4% bleach solution (Speare et al., 2004) or 30 sec in a 0.15% quaternary ammonium solution (Quat 128: http://www.fs.usda.gov/Internet/FSE_DOCUMENTS/fsbdev3_015333.pdf). Ideally, dedicated equipment should be used for a particular study site.

How to Detect and Describe Abnormalities.—There are many approaches to identifying morphological abnormalities in amphibians, including gross visual inspections (e.g., Ouellet et al., 1997), microscopy (e.g., Johnson et al., 2001b, 2002), radiographs of skeletal structure (Meteyer et al., 2000; Lannoo et al., 2003), clearing and staining to visualize skeletal structure (methods described in Dingerkus and Uhler, 1977; Kelly and Bryden, 1983; see also Sessions and Ruth, 1990; Kiesecker, 2002), dissections to inspect internal organs, and histology (e.g., Hayes et al., 2002). The choice of one or more of these methods will depend on the study objective. Overall, limb abnormalities have been the most common class of amphibian abnormality reported in the United States (Blaustein and Johnson, 2003; Ankley et al., 2004; Johnson

et al., 2010). Therefore, we discuss approaches to best identify, describe, and quantify limb abnormalities.

We advocate using visual field inspections to identify limb abnormalities because they (1) allow researchers to sample and release amphibians in a nonlethal manner, (2) are inexpensive to evaluate, and (3) provide immediate feedback to the researcher regarding abnormality status at the site. Although field-based inspections may miss internal and some external abnormalities, common limb abnormalities can often be observed with the naked eye and described in the field (USFWS, 2008). Laboratory-based techniques, such as those requiring stereo dissection microscope examination, radiography, or clearing and staining, can be used on previously identified abnormal amphibians to more thoroughly describe the bone or tissue abnormalities (Lannoo, 2008; Green et al., 2010). The use of laboratory-based inspection methods to determine the proportion of abnormal amphibians, however, has the disadvantage of requiring euthanasia of each inspected individual.

For every metamorphic amphibian examined, each limb and digit should be carefully inspected and examined for overall symmetry, with descriptions or photographs of each abnormality in the field. Some abnormalities (e.g., skin webbings) are only visible when limbs are manually extended. It is important to handle the animal gently, to keep one's hands or gloves moist while inspecting, to ensure that animals do not overheat on warm days. A detailed discussion of abnormality classification systems and terminology is beyond the scope of this paper but can be found in articles by Johnson et al. (2001b), Meteyer (2000), Meteyer et al. (2000), and especially in USFWS (2008).

A given individual may suffer from more than one abnormality, which has been termed abnormality severity, and can be calculated by summing the number of abnormalities on each abnormal amphibian (detailed in Johnson et al., 2001b). One drawback of this scoring system is that it weights extra limbs heavier than missing limbs; for instance, frogs with up to eight extra limbs have been observed, yet an individual can only be missing up to four limbs. Similarly, it is unlikely that a single missing digit is as severe as a completely missing limb, yet each are scored as one abnormality. This simple severity index could be improved by linking it more directly with fitness costs, for instance by incorporating the degree to which each abnormality type limits locomotion, increases risk of predation, or affects overall survival, when such data become available (e.g., Goodman and Johnson 2011).

How Many Malformations to Expect in a Population.—One of the most important challenges in studying amphibian abnormalities is determining whether observed malformations are outside the realm of what is expected in a given population. To draw a parallel with epidemiology, this is analogous to differentiating between "endemic" (or expected) and "epidemic" levels of a given disease. Such a dichotomous classification scheme involves determining whether the prevalence of abnormalities (total number of abnormal frogs / total number of frogs inspected) is within or greater than the expected baseline range. However, how the expected proportion of abnormal frogs in a population is defined can strongly influence data analyses and conclusions, especially when evaluating evidence of causation or whether malformations are increasing over time.

All populations of organisms can be expected to exhibit some morphological abnormalities resulting from genetic defects, developmental problems, and trauma. For example, in epidemiology, researchers determine whether a disease is above expected levels by comparing the disease prevalence in a given population to the endemic (background) levels (Merrill, 2009). If the disease prevalence is significantly greater than expected, it is classified as an "epidemic" in humans or an "epizootic" in animals (Merrill, 2009). We advocate using the same approach to determine whether an amphibian population exhibits an unusually high prevalence of abnormalities. However, such a comparison requires knowledge of the expected or baseline abnormality prevalence for amphibians. In humans, for example, survey data of more than one million births have found that the expected baseline level of congenital (at birth) limb malformations is 0.06–0.07% (Froster-Iskenius and Baird, 1989; McGuirk et al., 2001) and around 3% for overall birth defects (CDC, 2006).

Currently, the preponderance of data indicate that the expected morphological abnormality prevalence in recently metamorphosed anurans ranges between 2% and 5%, likely with some variation by geographic region, species under study, the type of abnormalities being considered, and the method of detection (Table 1). For example, 21 reference ponds in the Western United States had an average abnormality prevalence for *P. regilla* of 2.4% (data from Johnson et al., 2002). Large-scale field surveys across the United States and Canada have identified similar mean abnormality levels ranging from 0.3% to 4.3% (Table 1). In a review, Ouellet (2000) estimated baseline malformation prevalence between zero and 2% based on field studies with large sample sizes. One challenge in determining an unbiased baseline is that surveys across land use gradients will inevitably include some sites that are highly altered by agriculture, urbanization, or other anthropogenic stressors. These sites, therefore, may not be representative of the least-disturbed or reference wetland conditions. Further, differences in focal species, life stage, malformation classification system, and malformation scope (e.g., all morphology or only limb morphology) hinders the comparison across regions and species.

The U.S. Fish and Wildlife Service recently completed a 10-year survey of amphibian abnormalities across the nation using standardized monitoring of wetlands on National Wildlife Refuges (USFWS, unpubl. data). The USFWS survey sampled more than 48,000 anurans representing 409 sites, 37 species, and 41 states. This dataset represents the best available source of information to evaluate regional estimates of baseline malformation prevalence and to identify local areas or wetlands that exceed this baseline (see Reeves et al., 2008; USFWS, 2008, unpubl. data). This study identified a nationwide morphological abnormality prevalence of skeletal abnormalities averaging 2.0% (95% CI: 1.8–2.2). Some of the major types of skeletal abnormalities observed in this study were ectromelia (missing part of a limb), brachydactyly (short digit), and ectrodactyly (missing digit) (USFWS, unpubl. data). These data suggest that populations with significantly more abnormalities than this should be considered above-baseline. Yet, because of the variability in expected abnormality prevalence in the national survey by USFWS and other published studies as well as a lack of information as to how this figure might vary by region or species, we suggest a conservative threshold of 5% (~75th percentile) as a standard or expected baseline prevalence for limb malformations. It would be reasonable and prudent to modify the expected baseline level for a given survey based on locally derived species-specific data.

How to Determine Whether a Population Exhibits above-Baseline Levels of Malformations.—Continuing to adapt epidemiological methods to the amphibian malformation issue, we suggest that a

TABLE 1. Summary of recent multisite field surveys and museum studies of ambient abnormality prevalence. These surveys can be used to calculate a baseline or expected abnormality prevalence. Field surveys specifically investigating malformation hotspots or intentionally disturbed sites were not included in this table unless reference site data were provided.

Author(s)	Country	Region	Study year(s)	Species	Abnormality prevalence	Sample size	Site level data	Source
McCallum and Trauth, 2003	USA	Arkansas	1957–2000	*Acris crepitans*	7.1%	1,464	0 to 25%	Museum
Gray, 2000	USA	Illinois	1968–1971	*Acris crepitans*	0.4%	9,987	not provided	Field
Gillilland et al., 2001	USA	Michigan	1998	*Lithobates clamitans*[a]	0.3%	1,445	0 to 2.7%	Field
Converse et al., 2000	USA	Midwest, Northeast	1997	*Lithobates pipiens, L. clamitans*[a]	1.9%	8,899	0.4 to 15.6%	Field
Hoppe, 2000	USA	Minnesota	1996–1997	*Lithobates pipiens*	2.3%	2,548	0 to 3.7%	Field
Vandenlangenberg et al., 2003[c]	USA	Minnesota	1997–1999	*Lithobates pipiens*	4.3%	1,127	0 to 7%	Field
Hoppe, 2000	USA	Minnesota	1958–1963	*Lithobates pipiens*	0.7%	2,433	n/a	Museum
Schoff et al., 2003	USA	North Central US	1998–2000	*Lithobates pipiens*[a]	1.6%	2,605	0.8 to 8.8%	Field
Levey et al., 2003[c]	USA	Vermont	1997–2001	*Lithobates pipiens*[b]	3.4%	1,723	0 to 4.5%	Field
Taylor et al., 2005	USA	Vermont	2002	*Lithobates pipiens*[a]	1.6%	5,264	0 to 10.2%	Field
Johnson et al., 2002[c]	USA	Western US	1999	*Pseudacris regilla*	2.4%	1,722	0 to 10%	Field
Johnson and Lunde, 2005	USA	Western US	pre 1990	*Pseudacris regilla*	0.9%	658	n/a	Museum
Johnson and Lunde, 2005	USA	Western US	pre 1990	*Pseudacris regilla*	1.7%	1,328	n/a	Museum
Spolyarich et al., 2011	Australia	New South Whales	2005–2007	*Limnodynastes tasmaniensis, L. fletcheri, Litoria raniformis*	7.0%	1,209	not provided	Field
Eaton et al., 2004	Canada	Western Canada	1995–2002	*Lithobates sylvaticus*	0.6%	13,235	not provided	Field
Piha et al. 2006	Finland	South Finland	2002	*Rana temporaria*	1.0%	4,115	not provided	Field
Rostand, 1949 as summarized by Ouellet, 2000	France	France	not provided	*Bufo bufo*	1.0%	44,000	n/a	Field
Meyer-Rochow and Asashima, 1988	Japan	Japan	1981–1985	*Cynops pyrrhogaster*	4.8%	13,815	4.2 to 5.1%	Field
Huang et al., 2010	Taiwan	Taiwan	2006–2007	*Rana limnocharis, R. rugulosa*	2.2%	10,944	not provided	Field

[a]Focal species of study, but additional species were surveyed.
[b]Metamorphic frogs only.
[c]Summary of reference site data as defined by the authors.

statistical approach should be used to determine whether the sampled abnormality prevalence is greater than the suggested baseline prevalence (e.g., 5%). Statistical tests are necessary for this comparison because researchers are inferring population-level data (i.e., assuming abnormality prevalence for all individuals at the entire wetland) based on a small subsample of those amphibians (i.e., the number of individuals inspected). As a result of the dichotomous nature of the data (i.e., abnormal or normal), statistical confidence intervals around the prevalence estimate often follow a binomial or quasi-binomial distribution (Zar, 1999). Therefore, a Fisher's exact test, Chi-squared test, or *G*-test can provide simple and accessible estimates of whether the observed malformation prevalence is greater than the expected baseline range (Zar, 1999). If the lower 95% confidence interval exceeds 5%, the observed sample has a higher-than-baseline prevalence, and further investigations may be warranted to determine a site-specific cause. This is not to say that all wetlands with >5% abnormalities are necessarily a "problem" or that all of those with <5% are "healthy," but it does provide a well-defined, null hypothesis framework from which to build upon.

Issues with Malformation Prevalence: Sample Size, Statistical Confidence, and Bias.—Abnormality prevalence, calculated at each site and from each species, is a valuable and important measurement to collect during a field study. Sample size plays a pivotal role in determining the confidence of this estimate, whereby increasing sample size reduces uncertainty in the value.

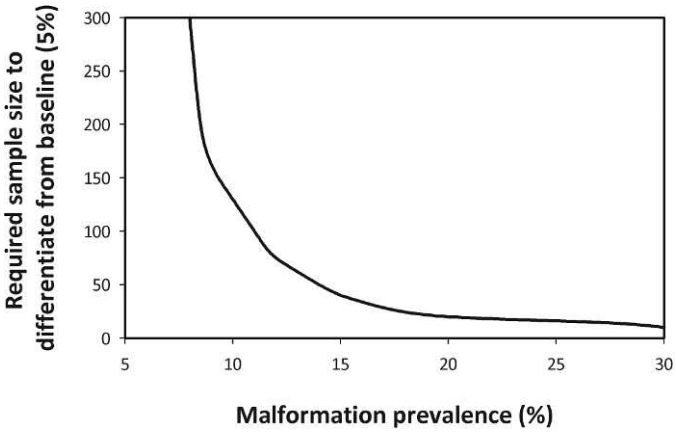

FIG. 1. Effects of amphibian sample size on a researcher's ability to reject the null hypothesis that an observed malformation frequency is within the "expected" baseline level of malformations (i.e., <5%) using a simple two-way Chi-squared test. With a sample size of 100 examined frogs, the observed malformation frequency must exceed ~12% to be statistically distinguishable from 5%, for example. Individual statistical comparisons can be made by calculating 95% confidence intervals of the prevalence estimate with statistical software packages such as JMP, STATA, or various web sites with binomial calculators, and determining whether the interval overlaps the expected baseline.

A simplified version of the relationships among sample size, malformation prevalence, and the probability of determining difference from baseline (i.e., statistical power) is illustrated in Figure 1. Sampling 100 amphibians, for example, allows for statistical comparisons to baseline levels (i.e., >5%) if the sampled malformation prevalence is 12% or greater. In contrast, if only 30 amphibians are sampled, the sampled malformation prevalence must be 17% or greater to be statistically differentiated from 5%. The same statistical tests can also be used to look for differences in malformation prevalence over time or to make comparisons among sites. In such comparisons, however, the data need to be from the same species and life stage and may require sample sizes larger than 100 if looking for small effect sizes.

For data on malformation prevalence to be useful, they must be collected and analyzed at the relevant spatial scale, which is often at the individual wetland, pond, or lake. Maintaining site-specific data is crucial to successfully discovering which sites have higher-than-baseline abnormality prevalence and identifying the cause(s) of the abnormalities at such sites. Datasets in which field observations from numerous sites have been combined together may fail to identify sites warranting additional study or provide insights about potential causes. For example, if a regional survey sampling 100 frogs per wetland discovered one pond with 30% prevalence and nine with 1% but proceeded to combine all the data together, the overall prevalence would be 3.9%, which is close to the expected baseline. Thus, the one hotspot and the chance to screen potential causes at that site would be missed.

Independent of statistical power, malformation prevalence can be influenced by sampling bias, potentially leading to inaccurate estimates. For example, field researchers may catch malformed frogs more easily compared to normal frogs, thereby inflating the prevalence estimate. Alternatively, malformed frogs might be removed selectively by natural predators prior to the sampling event; thus, the current population of malformed individuals would be an underestimate of the true prevalence. These challenges have caused some researchers to suggest that, because of potential errors in estimating malformation prevalence, it should not be used to study the issue (e.g., Lannoo, 2008). We disagree with this conclusion because malformation prevalence is one important piece of information for exploring whether a site has higher-than-expected numbers of malformations and, correspondingly, whether further study is warranted (USFWS, 2008). As with any sampling bias in ecology, researchers should use techniques to minimize potential biases and quantify existing uncertainties by statistically accounting for them in abnormality prevalence estimates.

Determining the Causes of Amphibian Abnormalities

Establishing that a parasite, pesticide, predator, or other factor is the proximate cause (etiologic agent) of observed abnormalities at a wetland often necessitates conducting experiments in which the hypothesized factor(s) is removed experimentally from all or part of the wetland. Researchers have successfully conducted in situ field studies to determine the major cause of malformations for *Ribeiroia* exposure (Kiesecker, 2002; Lunde et al., 2012; Roberts and Dickinson, 2012), as well as abnormalities resulting from stickleback fish predators (Bowerman et al., 2010). However, this work often takes years to complete at a single wetland and may be too time consuming and expensive to be conducted at the many sites with above-baseline abnormality prevalence. Further, simple exclosure or removal studies cannot be easily used to test for interactions among agents. Because of these limitations, we recommend adapting tools from disease ecology to best determine causation on a site-by-site basis.

Use of Multiple Lines of Evidence.—The use of "causal inference" is a valuable approach to investigate direct causes and indirect drivers of disease emergence (Plowright et al., 2008). Causal inference relies on using multiple tools (i.e., field correlations, long-term monitoring, causal diagrams, statistical modeling, dose-response relationships, nested field and laboratory experiments) to investigate complex interactions between ecological drivers and disease emergence. This approach is accomplished by using multiple lines of evidence to support causal relationships as opposed to experiments designed to test only a single hypothesis. Such research is best done through interdisciplinary cooperation, especially with an issue as complex as amphibian malformations, and should involve a combination of field and laboratory-based studies of developmental biology, herpetology, toxicology, physiology, and disease ecology. The adaptation of causal inference to determine the influence of a potential agent as the cause of malformations should include at minimum (1) detection of the agent at an affected site at or above levels shown to induce malformations in laboratory settings, (2) experimental studies that demonstrate the candidate agent can cause similar types and frequencies of abnormalities in relevant amphibian species, and (3) an association between the presence or abundance of the agent in nature and the occurrence of such malformations.

Interpretation of Dose-Response Relationships.—The first step in determining causation is detecting the potential abnormality inducing agent (whether chemical, fish, parasite, or other) at similar levels that have been shown to cause abnormalities or malformations in experimental studies. Such information may rely on dose-response relationships from laboratory studies or from field experiments (e.g., Johnson et al., 1999). It is important to consider, however, that dose response relationships for a single agent will vary depending on the amphibian species, as is the case for *Ribeiroia* infection (Johnson et al., 2010, 2012). Further, laboratory-based results do not always "scale-up" to the ecosystem level because of the simplicity required in laboratory experiments versus the complexity found in nature (Lunde et al., 2012). For example, pesticide exposure can sometimes weaken immune responses in frogs, increasing infection by trematode parasites (Kiesecker, 2002; Rohr et al., 2008a,b). In addition, field exposures to teratogens (agents that cause malformations) may occur at different times in larval development, especially if multiple species are present at the wetland. *Ribeiroia*, for example, only induces limb malformations during particular stages of limb development (Schotthoefer et al., 2003; Johnson et al. 2011). Thus, if the majority of a populations' parasite infection occurred in later stages, the site will appear below a dose-response curve generated from animals exposed at earlier stages in the laboratory.

Comparison of Malformation Compositions.—Given that multiple factors cause amphibian abnormalities in nature and that it is not typically feasible to conduct intensive field experiments at the many wetlands with above-baseline levels of abnormalities, there is a desire to differentiate among causes using field-derived malformation data. Thus far, researchers have used the types of abnormalities observed to infer causative agents among sites, similar to classifying diagnostic signatures for human diseases. For instance, Sessions et al. (1999) provided evidence that the type of limb duplication observed (proximal-distal axis vs. anterior-posterior axis) could be used to differentiate between

exposure to retinoids or mechanical perturbation, respectively. Such precise diagnostics, however, are often difficult to apply to amphibian abnormalities because a single agent can produce multiple classes or types of abnormalities (e.g., *Ribeiroia* produces both extra and missing limbs in larval amphibians), and a single malformation type can be produced by multiple different agents. Partially missing limbs (hemimelia), for example, can be caused by aquatic predators (Ballengée and Sessions, 2009; Bowerman et al., 2010), UV-B exposure (Ankley et al., 2000), and parasites (Johnson et al., 1999, 2001a, 2006). Thus, the presence of an individual malformation type is unlikely to be diagnostic of the factor that caused them in nature.

Nevertheless, the relative proportion of malformation types can be used to help determine the etiology or cause of the malformations when a causative agent is known to be present, particularly when there are experimental data illustrating the malformation response resulting from specific factors. For example, predators generally cause partially missing hind limbs or missing feet (Ballengée and Sessions, 2009; Bowerman et al., 2010). Likewise, UV-B radiation caused predominantly missing limbs but often in a bilaterally symmetric pattern (Ankley et al., 2000). In the case of *Ribeiroia* exposure, the types of malformations can vary among amphibian species (Johnson et al., 2012). For example, Pacific Chorus Frogs exposed to *Ribeiroia* experimentally develop an average of 55% extra hind limbs and 20% missing limbs (Johnson et al., 1999). In Leopard Frogs (*L. pipiens*), the malformation response to *Ribeiroia* was also exclusive to hind limbs but was mostly extra digits (25.6%) and extra hind limbs (22.2%) (Schotthoefer et al., 2003). In contrast, *Ribeiroia* exposure in Western Toads (*Anaxyrus boreas*) induced some fore-limb malformations (8%), and among both *A. boreas* and American Toads (*Anaxyrus americanus*) *Ribeiroia* caused predominantly skin webbings (34.4%), whereas extra limbs were less common (16%) (Johnson et al., 2001a; Johnson and Hartson, 2009).

The relative frequencies of malformation types are valuable data to establish causality at a site with above-baseline levels of malformations. When a sufficient number of malformed amphibians are detected at a wetland, generally 15–30 individuals, the species-specific malformation signature may be compared statistically to laboratory results or field experiments with specific causal agents. Statistical comparisons provide a transparent and unbiased tool to determine whether the types of malformations observed match a potential cause that is present at the wetland and is a substantial improvement from just comparing whether extra or missing limbs are found. Useful statistical tests for this comparison include quantitative similarity indices such as percent similarity or Bray-Curtis D (Boyle et al., 1990; Johnson et al., 2002). For example, malformation composition among *P. regilla* frogs at wetlands with *Ribeiroia* had a 70% similarity value when compared to results from experimental studies (Johnson et al., 2002). In contrast, similarities of $<21\%$ were observed when comparing *Ribeiroia* induced malformations and abnormality composition among anurans at sites without *Ribeiroia* (Johnson et al., 2002).

Although similarity indices and other statistical tools could be promising approaches for helping to identify or potentially eliminate hypothesized causes, there are limitations to this approach. For example, background abnormalities (i.e., genetic, developmental) are present at all sites and could obscure this type of analysis. As discussed previously, malformation type may vary in response to dose and timing of exposure (Schotthoefer et al., 2003). Additionally, the approach requires species-specific response data for each agent and, unfortunately, laboratory-based malformation signatures for multiple species are not available for most malformation causes. Without such data, researchers may assume similar tendencies within genera or even family. Yet this needs to be done with caution because different genera within the same family have shown strong differences in response to *Ribeiroia* infection (Johnson and Hartson, 2009). In agreement with Ankley et al. (2004), we recommend that laboratory tests for dose-response or malformation models use affected native amphibian species and recommend against using *Xenopus laevis*, an amphibian native to Africa, considering that *Ribeiroia* infection and pesticides are known to have species-specific effects (Degitz et al., 2000).

Methods to Screen for Trematode (*Ribeiroia ondatrae*) Presence and Abundance

As a detailed case example, we offer a description of how to find and identify one well-studied cause of limb malformations: infection by the trematode *Ribeiroia ondatrae*. Notably, *Ribeiroia* can be quickly assessed for presence and abundance using inexpensive tools available in most laboratories, making it an ideal candidate for a rapid screening procedure at sites with above-baseline malformations. If *Ribeiroia* metacercariae (the encysted stage of the parasite) are found in amphibian hosts at abundances known to induce malformations in laboratory studies, then relative malformation composition for each affected species can be compared to the malformation response identified in laboratory studies, and the role of the parasite as a causal agent can begin to be evaluated. If, however, *Ribeiroia* is present only at very low infection intensities (e.g., 1–2 metacercariae cysts detected per frog) and the malformation signature differs substantially from laboratory studies, then it is probable that another factor is causing the majority of the malformations (see Kupferberg et al., 2009; Bowerman et al., 2010).

In this manner, the presence or absence of *Ribeiroia* infection at a given site can be extremely informative. However, adequate screening for *Ribeiroia* requires a background in the parasite's life history. *Ribeiroia* is a multihost parasite, using aquatic snails as first intermediate hosts, amphibians as second intermediate hosts, and birds or mammals as definitive hosts (Johnson and McKenzie, 2008). Infected snail hosts produce cercariae, which are a free-swimming stage of the parasite infectious to larval amphibians. Because infection prevalence is generally much higher in amphibian hosts (50–100%) compared to snail hosts (1–5%) at the same sites, we suggest using amphibian hosts to screen for *Ribeiroia* presence and to use infection load in amphibians as a measure of the average parasite abundance (see below). Snail intermediate hosts can be useful for quantifying host-parasite dynamics, but they require more time intensive field and laboratory methods. However, if the amphibian species under study is threatened or a priority of the study involves avoiding the sacrifice of a vertebrate host, examination of snail hosts may provide a superior alternative.

Finding and Identifying Ribeiroia in Amphibians.—Inspections for *Ribeiroia* should be conducted on the same species and life-history stage as surveyed for malformation prevalence. Once again, metamorphic amphibians are the best indicator for several reasons: they are seasonally abundant; the removal of this life stage has reduced population impacts compared to adults; they are cumulative indicators of parasite exposure over the course of their larval periods; and they are very likely to be present at their

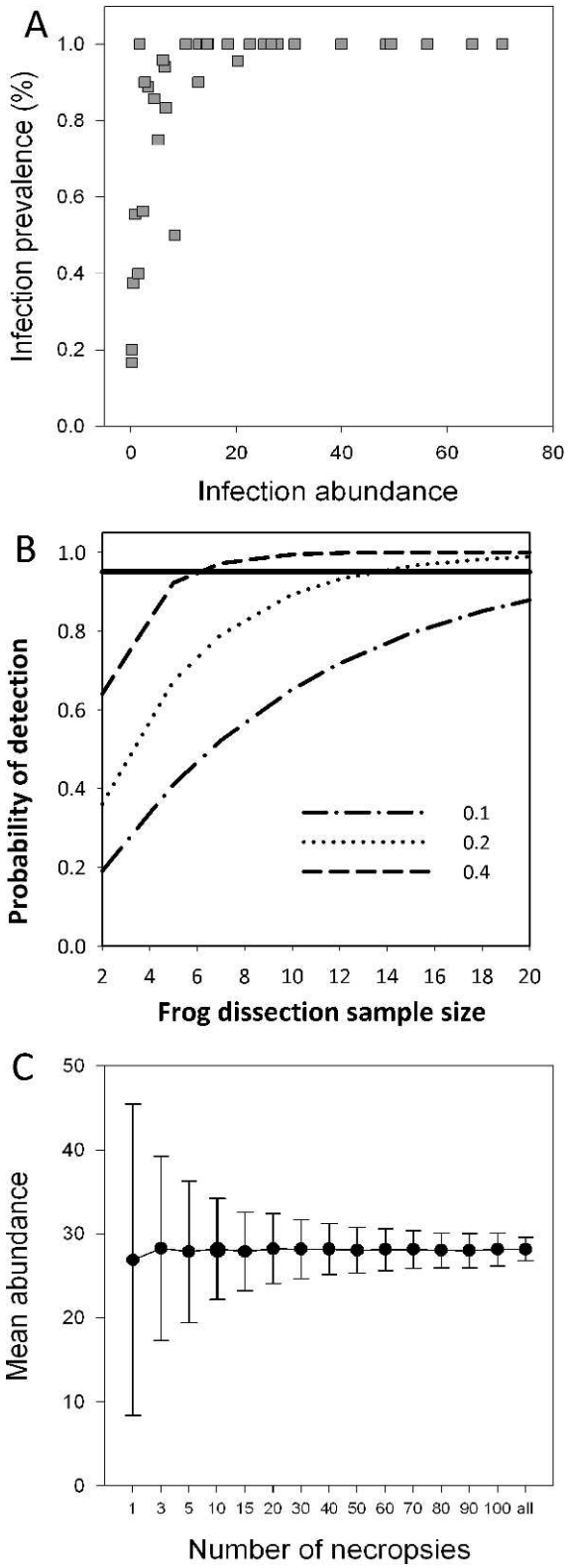

FIG. 2. (A) Relationship between *Ribeiroia* infection prevalence (% of infected frogs) and abundance (number of metacercariae per frog) in Pacific Chorus Frogs from California (2004–07). Prevalence increases rapidly with infection abundance, such that most frogs are infected at all but the lowest abundances of *Ribeiroia*. (B) Probability of detecting *Ribeiroia* in metamorphic frogs at 10, 20, and 40% infection prevalence in frogs. A sample size of 15 dissected frogs has a 96% probability of detecting *Ribeiroia* when it occurs at 20% (0.2) infection prevalence, which was the lowest prevalence observed in field-collected samples from Fig. 2A. The solid horizontal line represents a 95% probability of detection, a desired level of statistical power. (C) Relationship between the number of necropsied frogs and the estimate of *Ribeiroia* abundance. Error bars represent the standard deviation of the estimate following 1,000 resampling events. The "true" estimate of the mean was derived from 168 necropsies. Sample sizes of 10–15 frogs will generally have adequate error to make statistical comparisons between populations.

natal pond. Larval anurans might be sampled preferentially if two wetlands are close together and metamorphs may easily travel between sites or, if studying larvae that take more than one season to develop, such as the Green Frog (*Lithobates clamitans*), or American Bullfrog (*Lithobates catesbeianus*). When *Ribeiroia* is present at levels that can cause malformations, infection prevalence in small ponds often approaches 50–100%, such that nearly every frog necropsied will be infected and parasite detectability is very high (Fig. 2A). However, when infection prevalence is extremely low, for instance in <20% of the amphibians, a sample size of >15 frogs is required to have a >95% probability of detecting the parasite in at least one frog (Fig. 2B). For this reason, dissection of 10–15 frogs helps to limit the likelihood of missing *Ribeiroia* when it is present to less than 5% in most cases (Johnson and Hoverman, 2012). *Ribeiroia* intensity (number of cysts/infected individual) or abundance (number of cysts/individuals dissected) has adequate 95% confidence limits for most statistical comparisons when 15–20 amphibians are dissected (Fig. 2C), but a researcher's optimal sample size will depend on the expected difference in infection abundance and variance within the specific population (Bush et al., 1997).

To document the occurrence and abundance of *Ribeiroia*, amphibians should be dissected following standardized parasitological procedures. The most reliable method of identifying *Ribeiroia* involves dissecting a recently euthanized host, identifying live parasites via microscopy, and viewing the esophageal diverticula of *Ribeiroia*, which is the diagnostic feature for this species, with a compound microscope (Fig. 3) (Beaver, 1939; Yamaguti, 1971; Schell, 1985; Stopper et al., 2002; Johnson et al., 2004; Sutherland 2005; Szuroczki and Richardson, 2009). *Ribeiroia* metacercariae are generally found just under the host skin and above the skeletal muscles around limb structures, tail resorption sites in anurans, and the lower mandible (Sutherland, 2005). Cysts are 300–350 μm in size, appear clear or brownish in color, and can be isolated using fine-tipped forceps (Johnson et al., 2004). Cysts can be identified as *Ribeiroia* or other species on a compound microscope after breaking the cyst wall by gently applying pressure on the cover slip (Fig. 3). Detailed identification methods for *Ribeiroia* are described in other publications (Johnson et al., 2004; Sutherland, 2005; Szuroczki and Richardson, 2009).

The clearing and staining technique developed to visualize bones through skin and organs (e.g., Sessions and Ruth, 1990; Kiesecker, 2002) can be helpful in quantifying trematode parasite infection but will not always allow definitive identification of encysted parasites, of which there can be 10 or more species within a single amphibian host. Lentic amphibians are commonly infected with many species of digenetic trematode with cysts found under the skin such as *Alaria* spp., *Cephalogonimus* spp., *Fibricola* spp., and *Manodistomum syntomentera* (Sutherland, 2005), yet thus far only *Ribeiroia* is known to cause limb malformations in North America (but see Rajakaruna et al.,

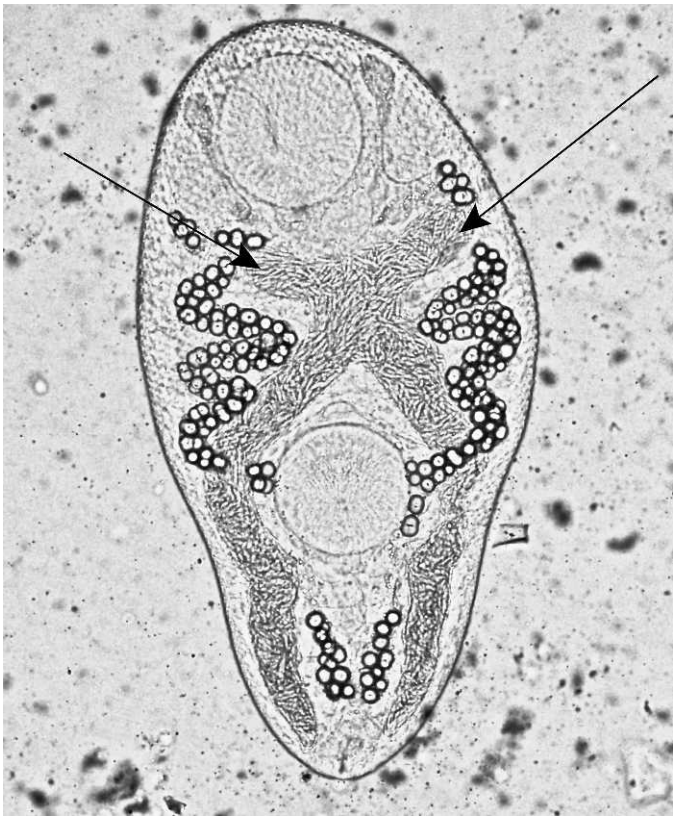

FIG. 3. Photograph of *Ribeiroia ondatrae* excysted metacercariae (opened cyst) isolated from an amphibian host. Esophageal diverticula noted with two arrows. Oral sucker is located at the top of the image, and ventral sucker is toward the bottom.

2008). Moreover, including all metacercarial cysts from multiple parasite species into an aggregate measure (e.g., total metacercarial abundance) is likely to obscure relevant trends related to *Ribeiroia* because each parasite species uses different snail and definitive hosts.

Finding and Identifying Ribeiroia in Aquatic Snails.—Compared to amphibians, determination of the presence of *Ribeiroia* in aquatic snail hosts can be more difficult because of low infection prevalence in first intermediate hosts. For example, at a seasonal pond in Mendocino County, California, a 1% *Ribeiroia* infection prevalence among rams horn (*Helisoma trivolvis*) snails caused a 50% malformation frequency among the co-occurring Pacific Chorus Frog (*P. regilla*) population (Lunde et al., 2012). Every single frog was infected (100% infection prevalence) with at least 36 metacercariae per host and an average infection intensity of 70 cysts. Thus, detecting *Ribeiroia* in snail hosts requires large sample sizes and can produce false negatives (missing it when present) because of the low infection prevalence compared to frogs. A random sample of 100 snails collected from this pond, for instance, has an ~40% chance of failing to yield an infected host. If 200 snails are sampled, this false negative rate drops to 13.4%. A sample size of 300 snails is required to limit the false error rate to a level of 5%. In contrast, a sample of one frog would ensure *Ribeiroia* detection. This example illustrates why snails can be less effective indicators of *Ribeiroia* presence compared to amphibian hosts.

Thus far, 12 species of snails worldwide and 7 in North America are known to support infection by *Ribeiroia,* all of which are within the family Planorbidae, a group commonly referred to as the "rams horn" snails, and within three common genera *Helisoma*, *Planorbella,* or *Biomphalaria* (Fig. 4A; Johnson et al., 2004; Johnson and McKenzie, 2008). When snails are abundant, a quick search of the shoreline can often yield evidence of living snails or shells that can be identified to family or perhaps genus (Clarke, 1981; Burch, 1989; Dillon, 2000). However, exhaustive searching of snails along the shoreline and in the water can sometimes fail to detect the presence of snail hosts when present at low densities (Johnson et al., 2002). Therefore, an absence of the appropriate snail hosts from a single sample event should not be taken as proof of absence for these snail species nor for absence of *Ribeiroia*.

Quantifying infection prevalence among snail hosts is an important factor affecting *Ribeiroia* abundance and often requires intensive laboratory methods to estimate. The first step is to determine snail density at the wetland using replicated, fixed-area measurements within the littoral (shallow) zone of the lake, pond, or wetland. This can be done using a standard-sized dip net (net mesh 0.5–2 mm) or a trashcan with the bottom removed. All or a random subset of snails from these collections can then be screened for infection to find the number patent (cercariae-releasing) snails, followed by dissection of the remaining snails to determine the number of prepatent or immature infections (Schell, 1985). Identification of patent infections is best accomplished by isolating individual snails into small containers (e.g., 50-mL vials or small cups) filled with filtered pond water or commercial spring water. Many trematodes emerge during the day, but *Ribeiroia* is released at night, such that a full 24-h cycle is necessary to estimate daily production per snail (although some parasites live for <12 h in their free-living stage; thus, frequent checking of the water is

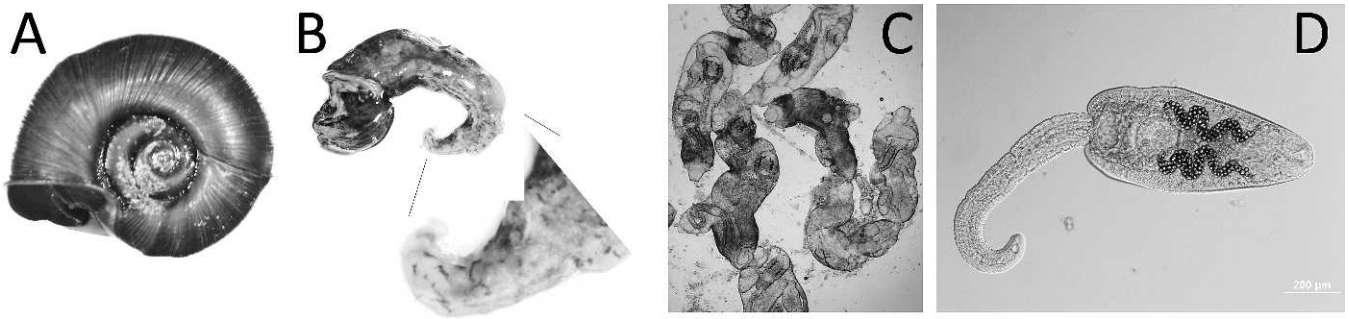

FIG. 4. (A) Rams horn snail in the family Planorbidae, which is the only family of freshwater snails known to host *Ribeiroia*. (B) *Ribeiroia*-infected rams horn snail, with inset of gonad tissue illustrating rediae that produce free-living cercariae infectious to amphibians. (C) *Ribeiroia* rediae under a compound microscope, which illustrates how partial and fully mature cercariae can be seen inside rediae during snail dissections. (D) *Ribeiroia* cercaria photographed with a compound microscope. Photo credit: Bryan LaFonte.

recommended). Patent infection status of each snail can be assayed by looking for free-swimming cercariae, which generally appear white against a dark background, a process that can be aided by using a strong overhead light source and a black backdrop. If cercariae are discovered in a vial, 3 to 4 can be pipetted onto a microscope slide and identified with a compound microscope using a larval identification key such as Schell (1985). *Ribeiroia* cercariae lack collar spines but possess esophageal diverticla (Fig. 4D; Stopper et al., 2002; Johnson et al., 2004; Szuroczki and Richardson, 2009). Staining of the cercariae with simple stains such as methylene blue can also facilitate identification (see Szuroczki and Richardson, 2009).

Snails that do not release cercariae can be dissected by crushing the snails with pliers and using forceps to tease apart the gonad region (the end deepest inside the shell) to look for rediae, sporocysts, or immature cercariae, any of which can be used to document a prepatent infection status (Fig. 4; Rohr et al., 2009). Although most cercariae in North America are not directly infectious to people, some can cause itching and irritation, and researchers are advised to wear gloves and protective eyewear during dissections of snails or amphibians. With immature infections, it is rarely possible to determine whether the parasite is in fact *Ribeiroia* or another morphologically similar parasite such as the common trematode *Echinostoma* spp. Molecular methods may be needed for identification in these cases (Reinitz et al., 2007).

Conclusions and Future Research

In this paper, we have presented some of the tools and information necessary to design and conduct malformations field surveys. Despite the growing body of knowledge regarding amphibian malformations and identified causes, many pressing questions remain. We highlight two pressing topics within the field of amphibian malformation study.

Identifying Important Causes of Malformations in Amphibian Populations.—Although a number of malformation hotspots across the United States have been associated with *Ribeiroia* infection, some wetlands with substantial numbers of malformations fail to support the trematode (Lannoo et al., 2003; Reeves et al., 2008). These sites are important places to discover the specific cause(s) of the malformations. Moreover, because the proximate cause of malformations often occurs in combination with other stressors, there is a need to examine additive or synergistic interactions between different factors. For example, exposure to *Ribeiroia* infection may occur alongside threats from chemical pollution, predators, or other pathogens, each of which have the potential to cause greater losses in amphibian populations (Koprivnikar et al., 2007; Rohr et al., 2008a,b). Kiesecker (2002), for example, found that experimental exposure to pesticides (Atrazine, Malathion, Esfenvalerate) reduced immune function that resulted in a threefold increase in *Ribeiroia* infection in Wood Frog larvae (*Lithobates sylvaticus*). Thiemann and Wassersug (2000) reported that the presence of predators significantly increased trematode infection in larval amphibians. *Ribeiroia* commonly occurs in ponds with dragonfly nymphs and fish predators (Ballengée and Sessions, 2009; Bowerman et al., 2010), highlighting that multiple agents can also act on the same amphibian species simultaneously.

Examining Conservation Implications Resulting from Malformations.—Amphibian populations and species are in decline worldwide and understanding whether malformations have the potential to contribute to ongoing declines is an important research priority. Declines have been attributed to habitat loss, invasive species, and emerging diseases such as amphibian chytridiomycosis (reviewed in Stuart et al., 2004; Skerratt et al., 2007; Wake and Vredenburg, 2008). However, no study has directly examined whether malformations pose a threat to amphibian populations, and this absence of evidence should not be taken as evidence that there is no connection. Multiple lines of evidence suggest that malformations have the potential to contribute to amphibian declines in areas where they are widespread. First, malformations impair the ability of frogs to jump, swim, and obtain food, and malformed frogs in nature exhibit 22% lower survival than normal conspecifics (Goodman and Johnson, 2011). Correspondingly, malformations are extremely rare in adult amphibians (<5%), even when abundant (>50%) in larval or metamorphic animals from the same wetland (Johnson et al., 1999; Lunde et al., 2012). Second, metamorphic amphibians with malformations may represent only a fraction of parasite-caused mortality with far more individuals dying as larvae, which is difficult to observe. Exposure to *Ribeiroia* is highly pathogenic and frequently causes substantial mortality in experimental studies (Johnson et al. 1999, 2001a, 2008; Stopper et al., 2002; Schotthoefer et al., 2003), and dead or dying tadpoles with hemorrhagic limb tissue characteristic of parasite exposure have been observed frequently at malformation hotspots. Thus, the total mortality as a consequence of *Ribeiroia* exposure, including direct death following infection and indirect losses associated with malformations, will be greater than the proportion of metamorphic frogs or toads that are malformed. Considering that species of amphibians declined or disappeared from Midwestern and Western malformation hotspots (Hoppe, 2002; Vandenlangenberg et al., 2003), we, along with others (e.g., Sessions, 2003), suggest that malformed amphibians represent a valid conservation issue. In particular, malformations found in rare or endangered species or in populations already threatened by other factors such as habitat loss, invasive species, and *Bd* infection, are of greatest concern.

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