16 A Decade of Deformities *Advances in Our Understanding of Amphibian Malformations and Their Implications*

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Abnormal amphibians have been reported in the literature for centuries. Since at least the 1700s, malformed amphibians, especially frogs with multiple hind limbs, have been documented in research articles, illustrated in atlases, and preserved in museums (Vallisneri 1706; de Superville 1740; Van Valen 1974; Ouellet 2000). Generally, such reports have involved an isolated individual or, in rare cases, a few animals. But it was not until August 1995, when a group of Minnesota middle school students discovered a pond in which 50% of the frogs had missing limbs, extra limbs or feet, bony protrusions, and skin webbings, that the issue of malformed amphibians was catapulted to the forefront of the public's attention (Kaiser 1997, 1999; Schmidt 1997; Souder 2000). Subsequent field surveys discovered large numbers of abnormal frogs in other regions of North America (NARCAM 2008), particularly parts of the Midwest (Helgen et al. 1998; Hoppe 2000, 2005; Vandenlangenberg et al. 2003), the West (Johnson et al. 1999, 2002, 2003), Alaska (Reeves et al. 2008), and the Northeast (Converse et al. 2000; Kiesecker 2002; Eaton-Poole et al. 2003; Levey 2003). The causes of observed abnormalities and their potential implications for human health and wildlife conservation became a topic of intense scientific debate and controversy. Now, more than a decade later, we revisit the issue and evaluate scientific progress in our understanding of amphibian malformations and their environmental significance.

16.1 BACKGROUND AND HISTORICAL CONTEXT

A thorough review of the historical literature on amphibian malformations can be found in the first edition of this book (Ouellet 2000). In the current edition, we build upon this historical foundation by incorporating recent advances in the study of malformed amphibians and addressing the implications of contemporary observations. We review laboratory and field data and propose questions that involve complex, large-scale issues of land use and environmental change. Throughout the chapter, we highlight existing data gaps and recommend areas that need further investigation. For a review of the patterns and mechanisms involved in limb development and disruption, readers are referred to other sources (Bryant et al. 1987; Gilbert 1997; Stopper et al. 2002).

Any investigation of amphibian malformations must begin by identifying what is "normal" or expected in an amphibian population, for which some terminology must also be defined. An "abnormality" is a general term referring to "any gross deviation from the normal range in morphological variation," and includes both "malformations" (permanent structural defects resulting from abnormal development) and "deformities" (alterations, such as amputation, to an otherwise correctly formed organ or structure; Johnson et al. 2001a). Abnormalities occur in all organisms, but what prevalence is considered normal for amphibians? Normal frequencies of abnormalities have been estimated at 0 to 2% (Ouellet 2000) and 0 to 5% (Johnson et al. (2001b). Recent studies looking at large numbers of frogs across Canada and the United States support a baseline frequency of 5% or lower: Minnesota (2.5%; Hoppe 2000), Michigan (0.14%; Gillilland and Muzzall 2002), the midwestern and northeastern United States (1.4% to 2.6%; Converse et al. 2000; Schoff et al. 2003), western Canada (0.2%; Eaton et al. 2004), Vermont (1.6%; Taylor et al. 2005), and Illinois (0.4%; Gray 2000). Surprisingly, many disagreements over the relative importance of potential causes of amphibian malformations can be explained by simple differences in how the "problem" is operationally defined. Thus, our focus here is explicitly on reports of abnormalities in amphibian populations that significantly and consistently exceed 5% (as determined by the 95% confidence interval around the estimate, which will be influenced by the percentage of malformed animals observed and by the total sample size examined). The use of a 5% threshold in conjunction with statistical confidence intervals may underestimate the number of amphibian populations or species exhibiting an abnormal level of malformations, but we believe this offers a rigorous and defensible approach for consistently defining the problem. While estimates of abnormality frequency are subject to various forms of bias (e.g., malformed frogs may be easier to catch and therefore overestimated, or may be quickly eliminated by predators and therefore underestimated), the use of a clearly defined "null hypothesis" of what is expected in a population allows sampling efforts to determine whether observed patterns differ statistically from what might be considered normal.

Several recent studies have reported much higher levels of malformations in newly metamorphosed frogs, ranging from just above baseline to greater than 50% (Helgen et al. 1998; Johnson et al. 1999, 2002, 2003; Converse et al. 2000; Kiesecker 2002; Eaton-Poole et al. 2003; Lannoo et al. 2003; McCallum and Trauth 2003; Vandenlangenberg et al. 2003; Hoppe 2000, 2005; Bacon et al. 2006; Gurushankara et al. 2007; Reeves et al. 2008). Observed abnormalities vary by site and by species but generally affect the limbs, including missing and partially missing limbs, irregular skin pigmentation, abnormal or deficient bone formation, extra limbs and limb elements, and a variety of other conditions that challenge conventional terminology (Figure 16.1). It is these sites and reports that have been the focus of more intensive studies to understand the factors responsible. Frogs with abnormal internal organs are rarely reported at high frequency and will not be emphasized in this review. This lack of information on internal malformations in wild frog populations is at least partially the result of difficulties inherent in documenting internal abnormalities without sacrificing large numbers of animals, which is not usually done in field investigations.

Historically, severe malformations are relatively uncommon in amphibian populations. Museum studies and resurveys of historic field sites suggest that, in some regions, abnormalities are increasing

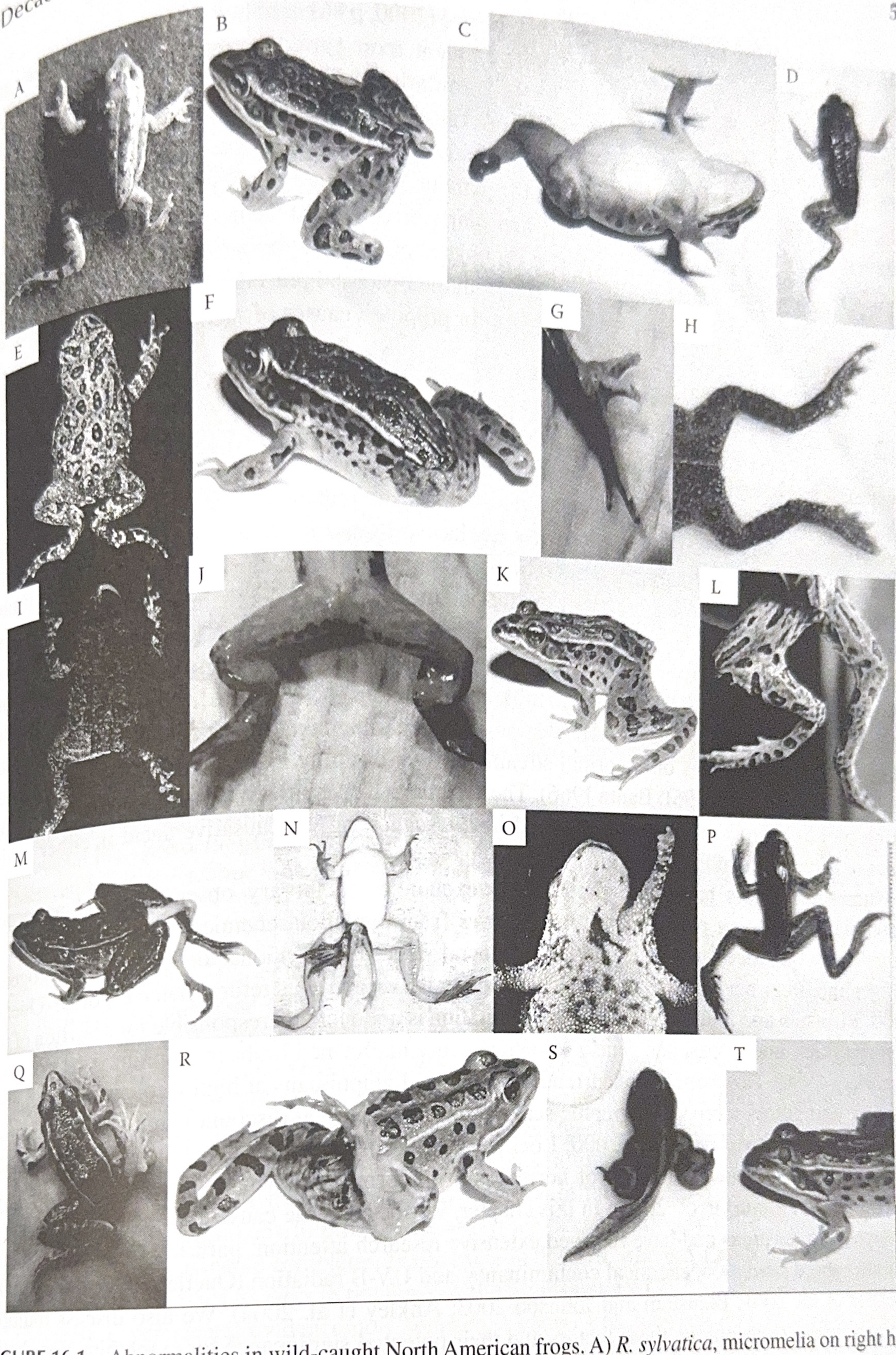


FIGURE 16.1 Abnormalities in wild-caught North American frogs. A) *R. sylvatica*, micromelia on right hind limb; B) *R. pipiens*, bilateral ectromelia; C) *R. pipiens*, apody on right hind limb and ectromelia at femur on left hind limb; D) *R. sylvatica*, ectromelia of right hind limb; E) *B. americanus*, ectromelia of left fore limb; F) *R. pipiens*, ectromelia of left hind limb; G) *R. pipiens*, syndactyly; H) *B. americanus*, brachydactyly of left hind limb; I) *B. americanus*, taumelia of right hind limb (note shortening of limb); J) *R. pipiens*, bilateral taumelia; K and L) *R. pipiens*, cutaneous fusion (skin webbing) between femur and calf on left hind limb; M) *R. pipiens* polymelia; N) *R. sphenocephala*, polymelia with extra ventral pelvis; O) *B. americanus*, polymelia of forelimb; P) *R. pipiens*, polydactyly with anteversion of left hind limb; Q) *R. pipiens*, taumelia and polydactyly of right hind limb; R) *R. pipiens*, hind limb malformation that challenges categorization; S) *R. draytonii*, bilateral edema of limbs; T) *R. pipiens*, anophthalamia of left eye. See Glossary at the end of the chapter for a description of terminology.

(Johnson et al. 2003). Working in Minnesota, Hoppe (2000, p 86) used both of these techniques and concluded that frog abnormalities were "more frequent, more varied, more severe, and more widely distributed in 1996 and 1997 than in 1958–93." Similarly, McCallum and Trauth (2003) reported a steady increase in amphibian abnormalities in Arkansas, from 3.3% (1957–1979) to 6.9% (1990s) to 8.5% in 2000. Johnson et al. (2003) noted that accounts of "mass malformations," in which >5% of the amphibian population exhibits limb malformations, are extraordinarily rare in the historical literature in the United States (1900–1990), particularly in contrast to the number reported since 1990 (e.g., NARCAM 2008). Collectively, these findings support the hypothesis that recently observed patterns of amphibian malformations deviate from the historical precedent. In the sections that follow, we review and evaluate available evidence for proposed causes of such malformations.

16.2 POSSIBLE CAUSES

Isolating the causes of abnormalities in amphibians is a necessary step in determining the implications of the phenomenon and the potential risks for both amphibians and humans. While no single cause can explain all deformities, several features have proven consistent. A review of the recent and historic cases yields the following observations: 1) abnormalities primarily involve the hind limbs, whether in the form of missing, extra, or malformed limbs (Hebard and Brunson 1963; Ouellet et al. 1997; Helgen et al. 1998; Hoppe 2005); 2) juvenile or premetamorphic frogs are the age class most commonly affected (Merrell 1969; Volpe 1977; Reynolds and Stephens 1984; Sessions and Ruth 1990; Johnson et al. 2001b, 2002); and 3) most abnormal individuals come from lentic or still-water habitats (Bishop 1947; Houck and Henderson 1953; Cunningham 1955; Hauver 1958; Lopez and Maxson 1990). Few cases of abnormal stream- or river-dwelling amphibians have been reported (see Cooper 1958; Ruth 1961; Banta 1966). These features direct our search toward causative agents acting on larval frogs with developing hind limbs, suggesting the causative agent is either in the water or affects amphibians while in an early aquatic stage.

Numerous factors have been proposed to explain contemporary observations of abnormal amphibians, including parasite infection, injuries from predation, chemical contaminants, UV-B radiation, ground-level ozone, radioactive mineral deposits, nutritional deficiencies, teratogenic viruses and fungi, acid precipitation, extreme temperatures, and even reformulated gasoline (Ouellet 2000; Blaustein and Johnson 2003). Genetic mutation is undoubtedly responsible for a portion of the abnormalities, and at least one gene is known to cause malformed limbs in amphibians (Droin and Fischberg 1980). However, the occurrence of abnormal amphibians at high frequency, in multiple species, and across a broad geographic scale has caused many to discount natural mutation as an important factor (Meteyer et al. 2000; Loeffler et al. 2001; Blaustein and Johnson 2003; Ankley et al. 2004), and genetic analyses of normal and malformed animals have revealed no signs of inbreeding (Williams et al. 2008). In this chapter, we focus on the causative agents that are likely to impact larger areas and have received extensive research attention: parasitic trematodes, injury and attempted predation, chemical contaminants, and UV-B radiation (Ouellet 2000; Cohen 2001; Loeffler et al. 2001; Blaustein and Johnson 2003; Ankley et al. 2004). We also discuss multiple stressor interactions among these factors and their potential significance for amphibian population viability.

16.2.1 Parasite Infection

Trematode parasite infection is probably the most thoroughly studied cause of amphibian limb malformations thus far. This hypothesis was first suggested by Sessions and Ruth (1990), who reported high frequencies of malformed Pacific chorus frogs (*Pseudacris regilla*) and long-toed salamanders (*Ambystoma macrodactylum croceum*) in a California pond. Upon clearing and staining specimens collected from the pond, Sessions and Ruth (1990) discovered high concentrations of trematode

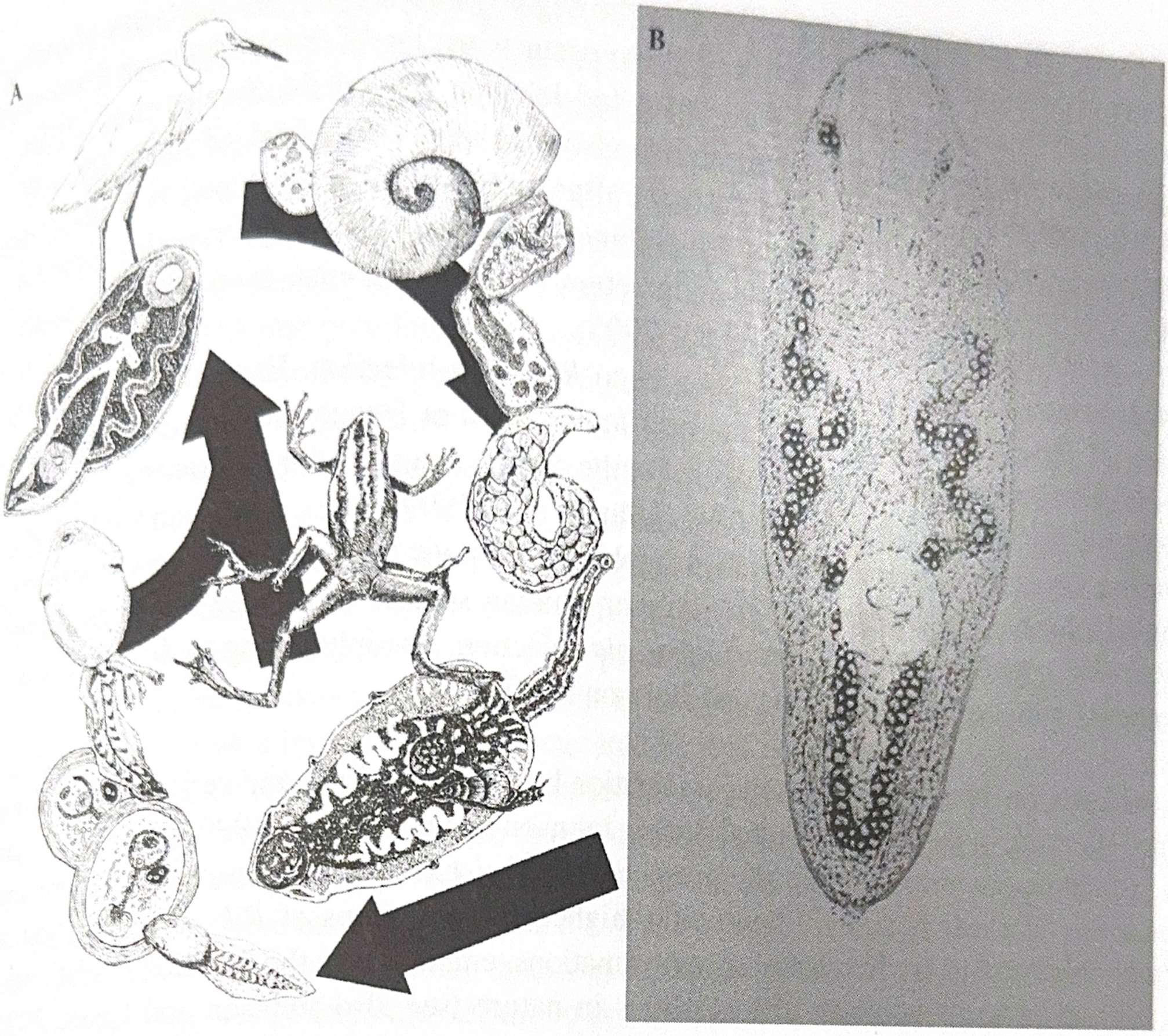


FIGURE 16.2 The digenetic trematode *Ribeiroia ondatrae* causes severe limb malformations in amphibians. A) The complex life-cycle of the parasitic trematode, *Riberoria ondatrae* (drawing courtesy of Brandon Ballengée); B) Metacercaria of *R. ondatrae* isolated from a malformed amphibian (image courtesy of D. Sutherland).

cysts around the limbs of affected individuals. Subsequent work later identified the parasite responsible as *Ribeiroia ondatrae* (Johnson et al. 1999, 2003), a digenetic trematode with a multihost life cycle (Figure 16.2). *Ribeiroia* moves sequentially among freshwater snails (rams horn snails in the genera *Planorbella* and *Helisoma*), larval amphibians, and finally, birds or, less frequently, mammals (Johnson et al. 2004). Infected snails release large numbers of mobile cercariae, which seek out and infect larval amphibians. Because cercariae specifically encyst around the developing limbs of amphibians, they can cause severe disruptions in limb growth leading to malformations. Interestingly, these malformations may aid transmission of the parasite by increasing the vulnerability of infected amphibians to bird predators, which become infected when they consume the amphibian host. The parasite completes its life cycle in the bird, wherein it develops into a sexually mature adult and releases its eggs in the feces of the bird (Figure 16.2).

Extensive experimental evidence now supports a causal link between *Ribeiroia* infection and limb malformations in amphibians. Exposure to realistic numbers of *Ribeiroia* cercariae causes increased mortality and severe malformations in species of frogs, toads, and salamanders (Johnson et al. 1999, 2001b, 2006; Kiesecker 2002; Stopper et al. 2002; Schotthoefer et al. 2003; Johnson and Hartson 2009). The frequency of malformations induced is often high, and can reach 100% among surviving animals. From this body of work have emerged several important patterns:

1) Malformations and mortality are dose dependent; higher levels of *Ribeiroia* exposure increase the risk and the severity of malformations produced, as well as the likelihood that animals die following exposure. Low levels of infection may or may not cause any obvious pathology.

2) The timing of parasite exposure influences the degree of pathology and the types of malformations. Malformations are most likely to occur when larval amphibians are exposed during early limb development (Bowerman and Johnson 2003; Schotthoefer et al. 2003). Exposure to *Ribeiroia* prior to limb growth also may increase the risk of mortality (e.g., Schotthoefer et al. 2003), whereas exposure after limbs are fully developed is unlikely to alter development. This suggests that amphibian species with differing larval development periods may exhibit different levels of infection or malformations, even within a single wetland (see Johnson et al. 2001a; Hoppe 2005).
3) Amphibian species vary in their responses to *Ribeiroia* infection. Exposure to identical levels of *Ribeiroia* infection may cause different types or frequencies of malformations in different species. For example, while Pacific chorus frogs exhibit predominantly extra limbs and digits in response to *Ribeiroia* (Johnson et al. 1999, 2002), American toads (*Bufo americanus*) suffer primarily from skin webbings and bony triangles, with very few extra limbs (Johnson and Hartson 2009). Some amphibian species (e.g., gray treefrogs [*Hyla versicolor*]) appear to be resistant to *Ribeiroia* infection, possibly owing to differences in innate immune response (Johnson and Hartson 2009).

Field evidence further substantiates the association between *Ribeiroia* and certain types of malformations. Working in the western United States, Johnson and colleagues (1999, 2002, 2003) have reported strong associations between the presence of *Ribeiroia* and the presence of severe limb malformations (significantly >5%). Importantly, higher concentrations of *Ribeiroia* infection are positively correlated with the frequency of malformations, emphasizing the functional relationship between parasitism and amphibian abnormalities in nature (see also Johnson and Chase 2004). Kiesecker (2002) reported similar patterns for a subset of wetlands in the Northeast, and showed that the experimental exclusion of *Ribeiroia* cercariae effectively eliminated malformations within field enclosures. *Ribeiroia* infection has also been linked to cases of severe malformations in the Midwest (e.g., Lannoo et al. 2003; Sutherland 2005; Johnson and Hartson 2009). It is important to emphasize, however, that not all methods used to detect parasites are equally effective. Clearing and staining of amphibians, which often involves removal of the skin and digestion of tissue, will often destroy parasites, leading to the false conclusion that *Ribeiroia* is absent (e.g., see Gardiner and Hoppe 1999 and Sutherland 2005 for contrasting findings for *Ribeiroia* infection in the "CWB" wetland in Minnesota). Examination of preserved frogs, frozen material, or histological sections can also obscure the identity of isolated parasites, as trematode metacercariae are often difficult to identify. Researchers should strive to examine freshly isolated, living parasites to detect key morphological features (see Sutherland 2005; Johnson and Hartson 2009).

Despite strong links between *Ribeiroia* infection and amphibian malformations in certain species and regions, *Ribeiroia* does not explain all accounts of malformations in amphibians. Some wetlands with a high frequency of abnormal frogs do not support *Ribeiroia* infection (Lannoo et al. 2003; Reeves et al. 2008). One of the advantages of trematode infection as a hypothesis to explain malformations is that it is very easy (and inexpensive) to test. If *Ribeiroia* is not found in amphibians following examination by a trained parasitologist, it is unlikely to be responsible for observed abnormalities. Using this method, researchers have eliminated *Ribeiroia* as a potential cause at several known malformation sites (see Lannoo et al. 2003; Linzey et al. 2003; Bacon et al. 2006; Reeves et al. 2008). If *Ribeiroia* is found in amphibians, it may or may not be responsible for frog abnormalities, depending on the amount of infection, the timing of infection, the amphibian species involved, and the types of abnormalities observed. For example, low levels of *Ribeiroia* or infection occurring outside of the limb development period are unlikely to cause malformations. In our experience, *Ribeiroia* is most commonly associated with specific types of malformations, such as skin webbings, bony triangles, shortened long bones, and missing or extra limb elements (Figure 16.1). However, extra limbs may or may not occur, depending on the amphibian species involved (see above). Thus, the recurring suggestion that *Ribeiroia* causes only (or even predominantly) extra

limbs is a gross oversimplification (e.g., Skelly et al. 2007). However, *Ribeiroia* is rarely associated with sites that produce exclusively missing limbs and/or digits in the absence of other malformation types, as reported at a number of wetlands (e.g., see Reeves et al. 2008).

Thus far, *Ribeiroia* is the only well-documented parasite to cause limb malformations in amphibians. Several other trematodes (e.g., *Alaria* and *Echinostoma*) failed to induce limb abnormalities in laboratory experiments (Fried et al. 1997; Johnson et al. 1999). However, growing evidence suggests that other parasites may also cause disruptions in limb development. For example, Rajakaruna et al. (2008) reported that an unidentified trematode (monostome type) caused missing and abnormal limbs in the common hourglass frog (*Hyla ebraccata*) in Sri Lanka. Whether such abnormalities also occurred at field sites with the parasite is unknown. Other parasites have also been linked to morphological abnormalities in amphibians. Murphy (1965) found that infections by an ectoparasitic mite were associated with limb and heart anomalies in pickerel frogs (*Rana palustris*). Similarly, Kupferberg et al. (2009) reported a significant association between ectoparasitic copepod infestations (*Lernaea* sp.) and limb abnormalities in foothill yellow-legged frogs (*Rana boylii*). Among larval and metamorphosing frogs, the prevalence of missing and abnormal limbs was 26% in copepod-infected individuals but only 1.1% in uninfected animals. Finally, Rostand and Darré (1969) suggested that a teratogenic virus excreted by fish was responsible for high rates of severe malformations observed over several decades in Europe (Ouellet 2000). When common frog (*Rana temporaria*) larvae were exposed to fish mucus during the first few days of development, digit malformations were produced (Surlève-Bazeille et al. 1969; Ouellet 2000). However, Surlève-Bazeille and Cambar (1969) were unable to produce the abnormality observed by Rostand and Darré (1969) when exposing *R. esculenta* to the fish mucus or bacterial cultures of it (Ouellet 2000). Whether the putative virus was ever isolated and identified is unclear, and to the best of our knowledge there has been little follow-up work by other researchers.

16.2.2 Predation

Predation is often cited as the single most important factor regulating amphibian survivorship and population size (Martof 1956; Calef 1973; Licht 1974; Cecil and Just 1979; Woodward 1983). Amphibian predators encompass a diverse collection of terrestrial and aquatic taxonomic groups, including mammals, birds, reptiles, fishes, aquatic insects, crayfish, and other amphibians (Martof 1956; Calef 1973; Licht 1974; Bradford 1989; Fauth 1990; Jennings et al. 1992; Kiesecker and Blaustein 1997). Tissue predation is almost certainly responsible for some fraction of observed abnormalities, but few studies have recorded baseline levels of injury in amphibian populations, making quantification of this component difficult (Martof 1956; Dubois 1979). To date, surprisingly little research has examined the potential role of predation in causing limb abnormalities, and predators are either ignored or discounted in most discussions of amphibian malformations (e.g., Lannoo 2008). The hypothesis implicating predation as a contributor to recent occurrences of amphibian abnormalities is often criticized on the grounds that: 1) a predator is far more likely to consume an entire frog rather than simply a leg; 2) injuries should produce scar tissue at the wound site, which would be an obvious indicator of earlier trauma in a metamorphic frog; and 3) a frog could not suffer an injury as severe as the loss of an entire limb without dying (Meteyer et al. 2000; Loeffler et al. 2001; Lannoo 2003, 2008). These assumptions may hold true for some predators and for injuries inflicted during certain periods, but there are many cases in which they are not supported. Although birds, mammals, turtles, and larger fish may consume an entire frog, smaller predators such as aquatic insects, small fish, and crayfish are much more likely to attack an exposed portion of an amphibian, such as a limb or a tail. Even more importantly, many of these predators will inflict injuries not on adult frogs, but on larval amphibians. Two important facts about predator attacks on larval amphibians are worth emphasizing. First, larval anurans have some capacity for tissue regeneration; thus, if the amputation or injury occurs during limb development, subsequent regeneration — while incomplete — is often sufficient to eliminate obvious

signs of trauma, creating a malformation (Fry 1966). Second, the loss of a tadpole limb — which is only partially ossified and incompletely vascularized — is likely to be far less severe than the loss of an adult frog limb. While many tadpoles likely die in response to such severe trauma (see Bohl 1997a, 1997b), those individuals that survive the injury to metamorphose can often exhibit missing or abnormal limbs.

Growing evidence now suggests that predators play an important role in causing certain types of abnormalities in amphibians. Recent experimental studies conducted with leeches, sticklebacks, and odonate naiads indicate that predation can cause a high frequency (e.g., >5%) of amphibian limb abnormalities. Two teams of researchers in Germany concluded that leeches (*Erpobdella octoculata*) were responsible for severe malformations in several populations of the common toad, *Bufo bufo* (Figure 16.3; Viertel and Veith 1992; Veith and Viertel 1993; Bohl 1997a, 1997b). In all cases, the abnormalities were dominated by partially and completely missing limbs, which exceeded 20% in some wetlands. While no genetic anomalies or contaminants could be linked to the abnormalities, experimental enclosures that permitted water but excluded predators eliminated the abnormalities in developing anurans (Bohl 1997a, 1997b). More importantly, investigators were able to reproduce the same suite of abnormalities in toads exposed to leech attack in the laboratory (Viertel and Veith 1992). While many larval toads died from the initial attack, those that escaped often developed abnormal limbs, similar to what was seen in field observations. Because anurans have partial regenerative ability

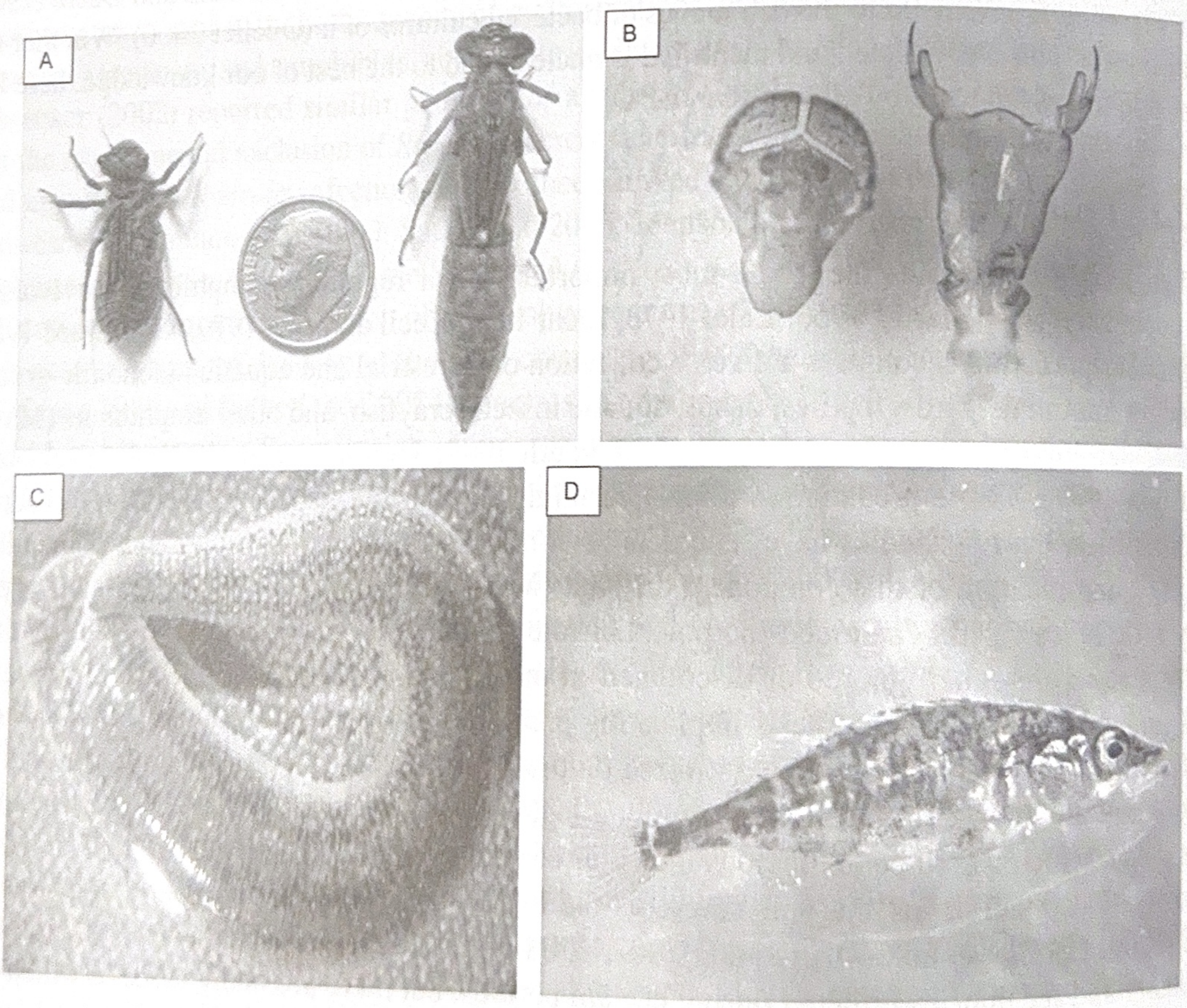


FIGURE 16.3 Aquatic predators capable of causing limb abnormalities. A) Two dragonfly larvae representing the families Libellulidae (left) and Aeshnidae (right), which both prey upon amphibian larvae (image courtesy of P. Jensen); B) Lower mandibles of libelluilid (left) and aeshnid (right) dragonfly larvae (image courtesy of J. Bowerman); C) Leeches (*Erpobdella* sp.) can attack and injure the developing limbs of tadpoles (Image copyright W. Moses, Smithsomian Institution, National Museum of Natural History, reprinted with permission); D) Three-spined sticklebacks (*Gasterosteus aculeatus*) have been linked to limb abnormalities in toads from Oregon (image courtesy of J. Bowerman).

prior to metamorphosis, some of the injuries became developmental malformations (also see Forsyth 1946; Fry 1966). Subsequent efforts to reduce the population of leeches within a wetland were successful in eliminating abnormalities in the toad population, offering compelling experimental evidence for the causal role of leeches (Bohl 1997b). Interestingly, however, because leeches were active primarily at night, investigators working during daylight did not initially realize their importance.

Similarly, Bowerman et al. (forthcoming) conducted a series of studies examining the importance of 3-spined sticklebacks (*Gasterosteus aculeatus*) and corduliid dragonfly larvae (*Somatochlora albicincta*) in causing amphibian abnormalities (Figure 16.3). Across a period of more than 10 years and >10 000 amphibians, they found that the annual abundance of non-native sticklebacks correlated positively with the frequency of limb abnormalities (missing limbs and digits) in western toads (*Bufo boreas*), which often exceeded 15%. As with the leech studies, experimental enclosures established within the lake precluded the occurrence of abnormalities in protected animals, while experimental laboratory trials revealed that sticklebacks will actively attack and injure the developing limbs of toad tadpoles, leading to abnormalities similar to those observed in the field (Bowerman et al. forthcoming). These same authors found that larval odonates (*Somatochlora albicincta*) can cause high levels (>15%) of missing and abnormal limbs in Cascades frogs (*Rana cascadae*). Through carefully designed experiments and sustained field observations, the authors established a link between the abundance of larval odonates and the types and frequencies of abnormalities observed among high-elevation wetlands in Oregon. On several occasions, odonates were observed attacking (and removing) the limbs of larval *R. cascadae*, and complementary experiments revealed that the addition of *S. albicincta* to enclosures induced the same types of abnormalities.

In the cases of leeches, sticklebacks, and larval insects, predators create an injury that either results directly in an abnormality (e.g., a missing limb) or creates a malformation during the regeneration process. This latter occurrence can be particularly important because the partially regenerated limb becomes a developmental malformation, even though it resulted from trauma. Literature on amphibian regeneration suggests that the timing and location of the injury are key determinants of the type of abnormality produced. Fry (1966), for example, found that if northern leopard frog (*R. pipiens*) limbs were amputated at early stages (i.e., prior to complete differentiation of the digits), these limbs regenerated into fully patterned albeit smaller limbs. When amputation occurred later in development, however, after joints and digits had already developed a pattern, the limb would heal into a stump lacking joints, toes, or other limb-like features (Fry 1966). More distal structures (feet and toes) retained regenerative ability longer than proximal structures (e.g., the femur). Reeves et al. (unpublished) found similar results for wood frogs (*Rana sylvatica*). Another study of 4 species of Japanese frogs produced comparable results: regenerative ability was present in all species, but varied among species and decreased with age at amputation (Kurabuchi and Inoue 1982). Taken together, these studies suggest that predator-mediated injuries early in tadpole development can cause abnormalities such as shrunken, missing, or truncated hind limbs, as documented in naturally occurring amphibian populations (see above and Reeves et al. 2008).

16.2.3 Biocides and Chemical Pollution

The hypothesis that anthropogenic chemicals in the environment are responsible for amphibian abnormalities is one of the most alarming. Amphibians are often cited as indicator species, showing heightened sensitivity to environmental conditions before they become deleterious for other species (Chandler and Marking 1975; Cooke 1981; Blaustein et al. 1994; Van der Schalie et al. 1999). Abnormalities in amphibians could represent a sublethal response to a toxic chemical in their habitat. If chemicals are the responsible agents, are they natural or anthropogenic, and what implications do abnormalities in amphibians have for human health and well-being?

Field and laboratory studies have explored whether chemical exposure plays a role in amphibian abnormalities. Field studies often focus on agricultural areas and compare abnormality prevalence in

areas with documented or presumed amphibian exposure to biocides with prevalence in "reference" areas. Laboratory experiments can be grouped into 2 categories: short-term tests with endpoints in the early-to mid tadpole stage (such as the 96-hour Frog Embryo Teratogenesis Assay–Xenopus [FETAX]) and tests with endpoints at the late tadpole stages (past hind limb emergence) or through metamorphosis. We assert that the most relevant laboratory studies for assessing possible causes of the types of abnormalities typically observed in the field are those that evaluate late-stage tadpoles or newly metamorphosed frogs. These studies should test a range of environmentally realistic and analytically documented contaminant concentrations.

In agricultural regions, amphibians are one of the nontarget groups most commonly affected by biocides, fertilizers, and their inert ingredients (Relyea 2005). This is largely because 1) the developmental timing of amphibian larvae often coincides with the season of heaviest biocide use, 2) the aquatic habitats used by amphibians may concentrate chemicals applied in the surrounding area, and 3) amphibian prey are one of the intended targets of a given biocide. Because of these spatial and temporal associations between biocides and breeding amphibians, agricultural chemicals and fertilizers have spurred numerous amphibian abnormality studies (see reviews in Ouellet 2000; Ankley et al. 2004).

Fieldwork has been an important component of investigations into agricultural and nonagricultural chemicals. Working in Canada, Ouellet et al. (1997) compared the frequency of anuran abnormalities on agricultural lands to those on nonagricultural lands. They recorded >10 times more abnormalities on agricultural sites. These results, while not statistically significant due to high variance, have been cited as suggestive and prompted additional studies. In western India, Gurushankara et al. (2007) found a higher prevalence of missing and shrunken limbs in rice paddies and coffee plantations than in forested areas used as reference sites. Taylor et al. (2005) found agricultural land use increased the risk of amphibian abnormalities in the northeastern United States. Piha et al. (2006), however, did not find such an association in agricultural areas in Finland.

Laboratory exposure studies have investigated the effects of agricultural chemicals on the prevalence of abnormalities. Here we summarize studies that noted external abnormalities. The many studies that examined whether exposure to atrazine results in gonadal abnormalities are summarized in Chapter 8. Grossly visible abnormalities have been induced by laboratory exposures to organochlorine compounds, including DDT (*Rana temporaria*, Cooke 1981), dieldrin (*Xenopus laevis, Rana catesbeiana*, Schuytema et al. 1991; *Rana perezi*, Alvarez et al. 1995), and lindane (*Xenopus laevis,* Marchal-Segault and Ramade 1981); organophosphates, such as folidol (*Rana perezi*, Alvarez et al. 1995), malathion (*Microhyla ornata,* Pawar et al. 1983), and guthion (*Xenopus laevis*, Schuytema et al. 1994); carbamates, such as ZZ-Aphox (*Rana perezi*, Honrubia et al. 1993, Alvarez et al. 1995), oxamyl (*Rana temporaria*, Cooke 1981), and carbaryl (*Rana perezi*, Bridges 2000); and certain herbicides, such as paraquat (*Rana pipiens,* Dial and Bauer 1984) and glyphosate (*Rana sylvatica*, Glaser 1998). Although the effects of these agricultural chemicals vary, it is clear they can cause skeletal abnormalities in developing amphibians. In laboratory experiments, carbamate and organophosphate pesticides caused scoliosis, shortening of the long bones, and twisted epiphyses in *Rana perezi* (Alvarez et al. 1995). The insecticide carbaryl caused 80% of exposed *Rana sphenocephala* tadpoles to develop abnormal curvature of the tail (Bridges 2000). The insecticides endosulfan and azinphosmethyl and the fungicide mancozeb caused skeletal and eye abnormalities in *Bufo americanus* and *Rana pipiens* (Harris et al. 2000). Recently, Brunelli et al. (2009) reported that endosulfan exposure of *Bufo bufo* from Gosner stage 25 (Gosner 1960) to metamorphosis resulted in an increased incidence of malformations, including bloated heads, kinked tails, and abnormal development of the mouth.

Nonagricultural pollutants have also been linked to amphibian abnormalities in the field. Amphibians exposed to coal combustion wastes exhibited a higher incidence of skeletal abnormalities in contaminated sites (18 to 37%) than in reference sites (0 to 4%; Hopkins et al. 1998, 2000). Flyaks and Borkin (2004) reported a high prevalence of hind limb abnormalities, including

polydactyly, reduction of limb segments, and asymmetric limbs in 3 anuran species (*Rana ridibunda*, *Bombina bombina*, and *Bufo viridis*) from the eastern Ukraine. Three areas were studied, 2 of which were highly industrialized. They measured tissue residues of metals and interpreted environmental data. The authors concluded that the frequency of abnormalities was correlated with the levels of environmental contamination.

A survey of cane toads, *Bufo marinus*, conducted from 2000 to 2003 in Bermuda, showed a high prevalence of limb abnormalities, particularly digit abnormalities and partially missing limbs (Bacon et al. 2006). To further investigate possible stressors, Fort et al. (2006a) conducted laboratory toxicity tests with *B. marinus* and *Xenopus laevis* using sediments and water collected from affected ponds. Chemical characterization of sediments indicated that metals, petroleum hydrocarbons, and ammonia occurred at potentially toxic concentrations. *B. marinus* embryos raised through metamorphosis in microcosms at the sediment-water interface exhibited abnormalities similar to those in free-ranging toads (Fort et al. 2006a, 2006b). Similarly, Sparling et al. (2006) conducted a dose-response study with *Rana sphenocephala* tadpoles using sediment from a shooting range spiked with concentrations of lead acetate. Surviving tadpoles demonstrated multiple skeletal malformations, including curvature of the spine and truncated and twisted femurs, long bones, and digits. All malformations were bilateral and essentially symmetrical, unlike most malformations observed in nature. Skeletal abnormalities occurred at concentrations of 540 mg/kg, far less than the highest contaminant concentration (5700 mg/kg) detected in wetlands near the range. Chen et al. (2006) also reported spinal deformities in northern leopard frog tadpoles exposed to lead in the water column. Scoliosis was detected in 92% of tadpoles exposed to 100 µg/L, and this condition was associated with abnormal swimming behavior. It persisted in some frogs after metamorphosis. A long-term laboratory exposure of green frogs (*Rana clamitans*) and northern leopard frogs (*Rana pipiens*) to PCB 126 through metamorphosis yielded few skeletal abnormalities (Rosenshield et al. 1999). Only the incidence of edema was significantly higher in both species exposed to the highest concentration (50 µg/L).

The fundamental challenge in investigating the role of contaminants in causing amphibian limb malformations in nature is determining which chemical(s) (or combinations thereof) to study. Testing for the full suite of these compounds and their various breakdown products in wetlands is prohibitively expensive and methodologically challenging. This has often caused investigators to focus their search on particular chemicals or classes of compounds. Beginning in the late 1990s, increased attention focused on the possible role of retinoids in causing amphibian malformations in nature (e.g., Gardiner and Hoppe 1999; Gardiner et al. 2003). Retinoids are vitamin A derivatives with well-documented tendencies to disrupt development (including limb growth) and pattern formation in vertebrates (Bryant et al. 1987; Bryant and Gardiner 1992; Maden 1993; Gilbert 1997; Gardiner and Hoppe 1999). The insect growth regulator methoprene, which was widely applied as an antimosquito agent and flea treatment for domestic pets (Harmon et al. 1995), initially received attention as a possible agent contributing to amphibian abnormalities because of the similarities between methoprene and retinoic acid (Henrick et al. 2002). Methoprene and its derivatives were initially found to cause developmental problems in *Xenopus* embryos even at low concentrations (Dumont et al. 1997; Degitz et al. 2000). Yet in later experiments, Degitz et al. (2003a) reported that the concentrations of methoprene required to cause developmental toxicity in amphibians were much more likely to cause mortality than developmental malformations (see also Ankley et al. 1998). Methoprene was also not correlated with the occurrence of amphibian malformations in nature (Sparling 2000; Henrick et al. 2002). Degitz et al. (2000, 2003b) arrived at a similar conclusion for the direct exposure of amphibians to exogenous retinoic acid. In experiments with both pulsed and continuous retinoic acid exposure, the authors reported that the conditions necessary to induce limb malformations in native amphibians were unlikely to occur in nature (see Ankley et al. 2004).

Building upon this foundation, Bridges et al. (2004) used a novel approach to investigate the possible role of contaminants in causing observed frog malformations. They deployed a series of semi-permeable membrane devices (SPMDs), which are integrated samplers that effectively "soak up"

lipophilic organic compounds from the environment, in 2 Minnesota wetlands: one with a history of severe malformations in multiple amphibian species ("impacted" site) and one with few observed abnormalities ("reference" site). After a 30-day deployment period, the authors retrieved the SPMDs from the ponds and raised leopard frog (*Rana pipiens*) larvae in water containing extracts from the SPMDs. Intriguingly, extracts from the impacted site caused a high frequency of bony triangles and skin webbings in metamorphosing frogs, leading the authors to suggest that a chemical or combination of chemicals from the pond was teratogenic. Extracts from the reference site did not cause bony triangles. However, skin webbings were observed in >40% of animals exposed to extracts from the reference site — which did not support these abnormalities in nature — and in 20% of animals in the SPMD control treatment, suggesting that more work is needed to understand the effects of this approach on amphibian development. Linking these results to field patterns is further complicated by the finding that the impacted site in this study supports extremely high abundances of *Ribeiroia* infection (see Sutherland 2005). Nevertheless, integrated approaches such as this that attempt to identify potentially teratogenic compounds from field sites have enormous potential in helping to narrow and direct the search for environmentally important contaminants.

In summary, while it is clear chemicals can cause skeletal malformations in amphibians, and field studies suggest chemical contamination is sometimes correlated with the occurrence of abnormalities, it has only been rarely demonstrated that chemical contaminants directly cause abnormalities similar to those observed under field conditions (Fort et al. 2001, 2006a, 2006b; Bridges et al. 2004). This issue is complicated by the diversity of chemicals released into the environment, the difficulties (and expense) in detecting parent and metabolite compounds in the field, and the proclivity for chemicals to interact with one another to affect amphibians. Future tests need to incorporate more ecologically relevant approaches that critically evaluate the effects of chemical agents on limb growth in native amphibians (see Degitz et al. 2003a, 2003b; Ankley et al. 2004; Bridges et al. 2004). Boone and James (2005) reviewed the use of mesocosms in amphibian ecotoxicology and cited several studies that have used mesocosms to evaluate the prevalence of abnormalities (e.g., Bishop et al. 2000; Harris et al. 2001; Kiesecker 2002). A promising approach is the coordinated use of field surveys, laboratory exposures, and mesocosm studies supported by tissue residue analyses (e.g., Flyaks and Borkin 2004; Boone and James 2005; Bacon et al. 2006; Fort et al. 2006a, 2006b).

16.2.4 UV-B Radiation

Declines in the earth's ozone layer have been connected with seasonal increases in the level of UV-B penetration, which is suspected to have deleterious effects on wildlife (Kerr and McElroy 1993; McKenzie et al. 1999; see Chapter 13, this volume). Because many amphibians deposit their relatively unprotected eggs in shallow water, this group is perhaps particularly vulnerable to changes in UV-B. Blaustein et al. (1994) demonstrated that ambient levels of UV-B radiation in the Oregon Cascades significantly reduced the hatching success of 2 amphibian species known to be in decline. In addition to mortality, developmental abnormalities were observed in surviving embryos exposed to solar UV-B radiation. Abnormal growth and development of the tail, concave curvature of the spine, bloating or distention of the body cavity, and improper development of the cornea were recorded in the embryos of multiple species (Hays et al. 1996). Subsequent studies have also noted adverse effects from laboratory UV-B exposures. Elevated UV-B increased mortality in larval and embryonic amphibians and caused developmental abnormalities, including skin burns, spinal curvature, abnormal cornea development, eye cataracts, and delayed development (Worrest and Kimeldorf 1976; Grant and Licht 1995; Ovaska et al. 1997). To specifically investigate the connection between UV-B radiation and amphibian abnormalities, Blaustein et al. (1997) conducted a field-based experiment exposing spotted salamander (*Ambystoma macrodactylum*) embryos to ambient UV-B conditions. Embryos protected from UV-B had a 95% survival rate with less than 1% abnormality rate. In the unshielded condition, hatching success was less than 15% and the

frequency of abnormalities in surviving embryos was 86%. The predominant abnormalities were lateral flexure of the tail, blistering of the skin, and edemas (Blaustein et al. 1997).

While these studies underscore the potential danger of UV-B exposure for embryonic and larval amphibians, they do not directly address the importance of UV-B in causing limb abnormalities. Limited evidence suggests that, in some circumstances, UV-B exposure can cause limb malformations. Butler and Blum (1963), for example, reported that localized UV irradiation (at greatly elevated levels) could induce supernumerary limbs in salamander larvae (see also Ankley et al. 2004). More recent laboratory and outdoor studies have established that exposure to ambient UV-B can cause high frequencies of limb reductions or deletions in amphibians (Ankley et al. 2000, 2002). However, the resulting abnormalities were generally bilaterally symmetrical, unlike most field observations, causing the authors to question a direct role of UV-B in explaining recently observed malformations in amphibians (Ankley et al. 2002, 2004). Additionally, extensive field studies and risk analyses suggest that the levels of UV-B exposure to which amphibians are exposed in natural wetlands will often be insufficient to induce abnormalities (Peterson et al. 2002; Diamond et al. 2002). In nature, UV-B is rapidly attenuated in aquatic ecosystems, often within a few centimeters, owing to dissolved organic carbon in water (Palen et al. 2002; Diamond et al. 2002; Ankley et al. 2004). Thus, while UV-B may pose a threat to amphibian eggs (which are laid in shallow water by some species) or in exceptionally clear or shallow water, most evidence suggests that the UV-B hypothesis is unsupported as a major cause of limb abnormalities in nature (Blaustein and Johnson 2003; Ankley et al. 2004; Reeves et al. 2008). UV-B radiation is more frequently associated with problems of the cornea, skin, or neural and spinal development (Malacinski et al. 1974; Blaustein et al. 1994, 1997; Grant and Licht 1995; Ovaska et al. 1997; Ankley et al. 2004).

16.3 MULTIPLICITY OF CAUSES

Given the wide range in the composition of abnormalities, the large number of amphibian species recorded with abnormalities, and the broad geographic range affected, we suggest that multiple factors are almost certainly responsible for the amphibian abnormalities documented since the early 1990s. The involvement of multiple causative agents heightens the complexity of the investigation considerably. Sites with similar abnormalities may, in fact, be affected by unrelated causes. Alternatively, multiple, interacting causes may be operating within a single wetland.

Whereas all factors currently under investigation (chemicals, parasites, predators, or UV-B radiation) have the potential to induce abnormalities, it is difficult to assess if they actually cause abnormalities in situ. Studies need to be carefully designed to detect multiple, potentially interacting causes. This process has been hindered by the artificial division of causes into "natural" (parasite, injury) and "anthropogenic" (elevated UV-B radiation, xenobiotic chemical) sources. Such categories are misleading because they ignore the very real potential for interactions among these factors and because they (wrongly) suggest that natural factors are unlikely to be a problem. For example, malformations caused by nonnative predators or by emerging pathogens may pose a very real threat to amphibian populations, despite that both agents are natural. To illustrate this point, consider the case of emerging pathogens in human and wildlife populations. While most of these pathogens are natural, they can nevertheless pose a significant threat to affected hosts, often owing to changes in infection prevalence caused by underlying environmental changes (e.g., Daszak et al. 2000; Harvell et al. 2002). We argue that the putative causes of abnormalities should instead be classified as either *biotic* or *abiotic* (see Johnson and Lunde 2005), and suggest that interactions between these groups are more likely to be the rule than the exception.

Several studies have found evidence for multiple interacting factors in driving amphibian abnormalities, particularly in association with *Ribeiroia* infection. Kiesecker (2002), for example, reported that larval wood frogs exposed to common pesticides developed higher levels of *Ribeiroia* infection than did control animals. Based on the reduced levels of circulating eosinophils in the

blood of amphibians exposed to pesticides, Kiesecker (2002) suggested that contaminant exposure reduced the immune response of amphibians in response to parasitic invasion, leading ultimately to higher infection levels (see also Belden and Kiesecker 2005; Koprivnikar et al. 2007). More recently, Johnson et al. (2007) showed that eutrophication resulting from nutrient runoff can increase the levels of *Ribeiroia* infection. Excess nutrients stemming from agricultural fertilizers, livestock manure, or urbanization enhance algal growth in aquatic systems, ultimately promoting the growth and reproduction of the snail hosts for *Ribeiroia* (e.g., *Planorbella* spp.). In a large-scale mesocosm experiment, Johnson et al. (2007) found that nutrient enrichment led to increases in both the density of infected snails and the per snail production of infectious cercariae. This increase in parasite production translated directly to an increase in *Ribeiroia* infection in larval amphibians. Rohr et al. (2008) recently found evidence that both nutrient runoff and pesticides were contributing to elevated trematode infections in Minnesota amphibians, underscoring the complexity of the relationships between environmental change and host-parasite interactions.

The presence and composition of predators in freshwater ecosystems can also indirectly influence trematode infection. Larval amphibians often change their behavior in response to the chemical cues from fish or dragonfly predators, spending more time immobile or hiding to avoid detection. Such changes may increase their vulnerability to parasite infection. Thiemann and Wassersug (2000) found that amphibian larvae exposed to chemical cues from caged predators spent less time moving and developed significantly higher trematode infections. In a related example, Johnson et al. (2006) found that conspecific attack (i.e., cannibalism) by amphibians can increase the frequency of parasite-induced malformations. Larval salamanders frequently attack one another, often causing limb injury or loss (e.g., Crump 1983; Walls et al. 1993; Wildy et al. 2001). If injured animals were subsequently exposed to *Ribeiroia* during the regeneration process, the limbs were 3 to 5 times more likely to develop permanent malformations than uninjured limbs. Predators, however, also have the potential to reduce infection. By consuming trematode cercariae, dragonfly larvae and other predators could indirectly reduce infection and malformations in amphibians (Schotthoefer et al. 2007; Thieltges et al. 2008).

Finally, environmental contaminants have the potential to interact with factors such as UV-B radiation, pH levels, predator cues, and other contaminants (e.g., Relyea and Hoverman 2006). For example, Relyea (2005) found that commercial-grade Roundup®, containing the active ingredient glyphosate and a polyethoxylated tallowamine (POEA) surfactant, exposure was far more lethal to amphibians when combined with the presence of predators (as would occur in nature) than under artificial laboratory conditions. Such interactions underscore the complexity of investigating amphibian malformation phenomena in nature. To summarize: 1) amphibian abnormalities can be caused by a variety of abiotic and biotic agents, 2) there is frequently more than one causal agent present, 3) these agents vary in importance among sites and years, and 4) no single cause is likely to explain all high-frequency accounts of amphibian deformities in the wild (Carey et al. 2003). A brief overview of the US Fish and Wildlife Service's Abnormal Amphibian Project illustrates the complexities inherent to a causal analysis focused on malformations in amphibians.

16.4 CASE STUDY: US FISH AND WILDLIFE SERVICE, ABNORMAL AMPHIBIAN PROJECT

In 2000, the US Fish and Wildlife Service (USFWS) began a uniquely large-scale and collaborative program focused on understanding the geographic distribution and severity of amphibian abnormalities on US National Wildlife Refuges. The objectives were to 1) determine the prevalence of abnormalities in frogs on refuges; 2) evaluate how abnormality frequencies vary among sites, refuges, and years; and 3) investigate possible causes of the abnormalities through targeted follow-up studies.

During the first 6 years of the study (2000 to 2005), a total of 46 530 frogs and toads were collected and examined, with the vast majority released after inspection (USFWS 2009). Between 2000 and 2005, 112 refuges in 46 states were sampled at least once for abnormal frogs, and many refuges were sampled on multiple occasions. A total of 3093 individuals (6.6%) were classified as

abnormal for various reasons, including injury or signs of disease. Of these, 1239 (3.1%) of the total were classified as having skeletal abnormalities.

A subset of the frogs was inspected for parasites and analyzed with radiography. Guderyahn (2006) summarized and interpreted the radiographic analysis of 666 abnormal frogs collected during the 2003 and 2004 field seasons from refuges within 27 US states. The objectives were to use radiography to provide diagnostic information on the nature of the abnormalities and to compare types and frequencies across regions. Despite considerable differences in species and ecological factors (e.g., habitat type, climate, and land use), abnormalities were generally similar across regions of the country. The most commonly affected body part was the hind limb (80% of the 861 abnormalities). Hind limb brachydactyly (shortened digits, 27%) and ectromelia (missing limb segments, 23%) were the most frequently observed abnormality types, followed by ectrodactyly (missing digit[s], 7%), hemimelia (shortened limb, 5%), and bony expansions (5%). Other hind limb abnormality types comprised <3% of all observed abnormalities.

Research comparing the types of abnormalities in different regions as well as what stressors are present is currently under way in an attempt to identify presumptive cause-effect relationships at refuges with elevated incidences of abnormalities. In several cases, in-depth studies were conducted on individual refuges or on a group of refuges. These studies are summarized in the following subsections.

16.4.1 Great Bay National Wildlife Refuge

Great Bay National Wildlife Refuge (Newington, New Hampshire) was created on the former Pease Air Force Base, listed as an EPA Superfund (CERCLA) hazardous waste site due to high concentrations of trichloroethylene and nitrate in ground water; pesticides, polycyclic aromatic hydrocarbons (PAHs), and metals in sediment; and pesticides (including DDE) in fish tissue. The Great Bay in-depth study (Pinkney et al. 2006) was triggered by observations of abnormalities greater than 9%, 2 amphibian die-offs, and contaminant concerns. These concerns included the results of 140-day laboratory tests using *Xenopus laevis*, which exhibited increased mortality and delayed metamorphosis when exposed to refuge pond sediment (Turley et al. 2003). Laboratory and in situ toxicity tests with wood frogs and northern leopard frogs, chemical sampling of water and sediments, and abnormality surveys were conducted at 4 ponds. Decreased survival and increased time to metamorphosis occurred in wood frogs exposed either in situ or in the laboratory to passive sampler extracts, water, or sediments from several refuge ponds. The major observation in the in situ study was the high prevalence of a leg abnormality (rounded femurs), which resulted in impaired hopping ability. This was observed in 63% of the wood frogs from the refuge's Beaver Pond, compared with 0 to 1% in wood frogs from the 3 other refuge ponds. This leg abnormality was confirmed in x-rays as a probable malformation. There was, however, no evidence of a linkage between adverse effects in the in situ study with exposure to DDT or other pesticides. Pinkney et al. (2006) recommended abnormality and population monitoring, maintenance and monitoring of water levels in frog habitats, and further sediment sampling. While present on the refuge, *Ribeiroia* was extremely rare in field-collected frogs and therefore unlikely to significantly contribute to observed malformation patterns.

16.4.2 Alaskan National Wildlife Refuges

A study of 5 Alaskan National Wildlife Refuges, conducted in tandem with the national abnormal amphibian project, has indicated a pattern in the abnormality frequencies. Reeves et al. (2008) examined 9269 metamorphic wood frogs from 86 breeding sites from 2000 through 2006. The prevalence of skeletal and eye abnormalities at Alaskan refuges ranged from 1.5% to 7.9% and was as high as 20% at individual sites. The most common types of abnormalities were ectromelia, micromelia (shrunken limb or limb element), and unpigmented iris. Proximity to roads (as a proxy for human development) significantly increased the likelihood of skeletal abnormalities and skeletal malformations but not eye abnormalities. The authors suggested either chemical contaminants,

invertebrate predators, or an interaction between these factors was causing the abnormalities. *Ribeiroia* was not detected among any of the necropsied frogs from the region, and overall parasite richness and abundance were both very low. Their data did not support the UV-B or parasite hypotheses for wood frog abnormalities in Alaska.

16.4.3 Parasitological Investigations on NWRs

From a subset of refuges sampled across the country, metamorphosing amphibians (normal and malformed, if present) were necropsied to identify and quantify their parasites. While the focus was on *Ribeiroia*, data were collected on all encountered helminths (parasitic worms), including other trematodes (flatworms), cestodes (tapeworms), nematodes (roundworms), acanthocephalans (spiny-headed worms), and various protistan (protozoan) parasites (Sutherland 2005). To the best of our knowledge, these data comprise one of the largest amphibian parasite databases ever assembled. Thus far, we have examined more than 22 species of amphibians from 40 refuges and 28 states. More than 200 000 individual parasites have been identified and enumerated.

While this sampling effort is ongoing and much of the data have yet to be analyzed, several interesting patterns have already emerged. *Ribeiroia* appears to be relatively widespread but is highly variable in abundance, both among refuges and between years within a refuge. Fifteen of the 40 refuges sampled for parasites tested positive for *Ribeiroia*, including infections in 8 different amphibian species. Infection within amphibians ranged from 1 to 177 parasites in an individual frog. *Ribeiroia* also exhibited distinctive distributional patterns across the United States. Interestingly, we have recovered *Ribeiroia* only in frogs from the northern half of the United States, with no records below 37° latitude (Figure 16.4). This is somewhat surprising given the preponderance of *Ribeiroia* records in birds from Florida (see Johnson et al. 2004). In addition, very few trematodes (and no records of *Ribeiroia*) have been found in Alaska (despite the significant occurrence of abnormalities; see Reeves et al. 2008), possibly owing to the difficulties inherent in parasite overwintering or to the very short growing season in which transmission must occur. More data are needed to understand these intriguing patterns.

Ribeiroia-positive refuges occurred primarily along the West Coast, in the Northeast, and in the Midwest along the Mississippi River (Figure 16.4). This pattern likely underscores the importance of the major migratory bird flyways, including the Pacific flyway, the Mississippi flyway, and the Atlantic flyway, and is therefore consistent with birds acting as the major transport vector for *Ribeiroia*. Why we have not found more records along the southern half of the migratory pathways is unclear. In a study of helminths in wood ducks (*Aix sponsa*), Thul et al. (1985) found that *Ribeiroia* was a dominant member of the helminth fauna in migratory ducks in the Northeast, with a prevalence of 21%, whereas *Ribeiroia* was rare (<2% prevalence) among resident wood ducks in the Southeast, similar to our current investigations with frogs.

In summary, *Ribeiroia* infection is widespread but variable among refuges in the United States. At high infection levels, *Ribeiroia* is associated with the occurrence of particular malformation types that vary in frequency among species. These malformations are generally dominated by skin webbings, bony triangles, and truncated long bones. Extra limbs and limb elements occur in some species but not in others. Sites without *Ribeiroia* often exhibit abnormalities involving primarily missing digits, limb elements, or entire limbs. While such abnormalities often occur at low frequencies, within the expected baseline levels, other refuges (such as those in Alaska) exhibit high frequencies (>10%) of abnormalities across multiple years. Thus far, however, the causes of these abnormalities remain unknown (see Reeves et al. 2008).

16.5 CONTINUED STUDY OF AMPHIBIAN ABNORMALITIES

Amphibian abnormalities have been the focus of extensive press and scientific debates since reports increased in the late 1990s. The confirmed accounts of abnormal amphibians in more than 30 states in the United States have created substantial concern that abnormalities indicate the presence of

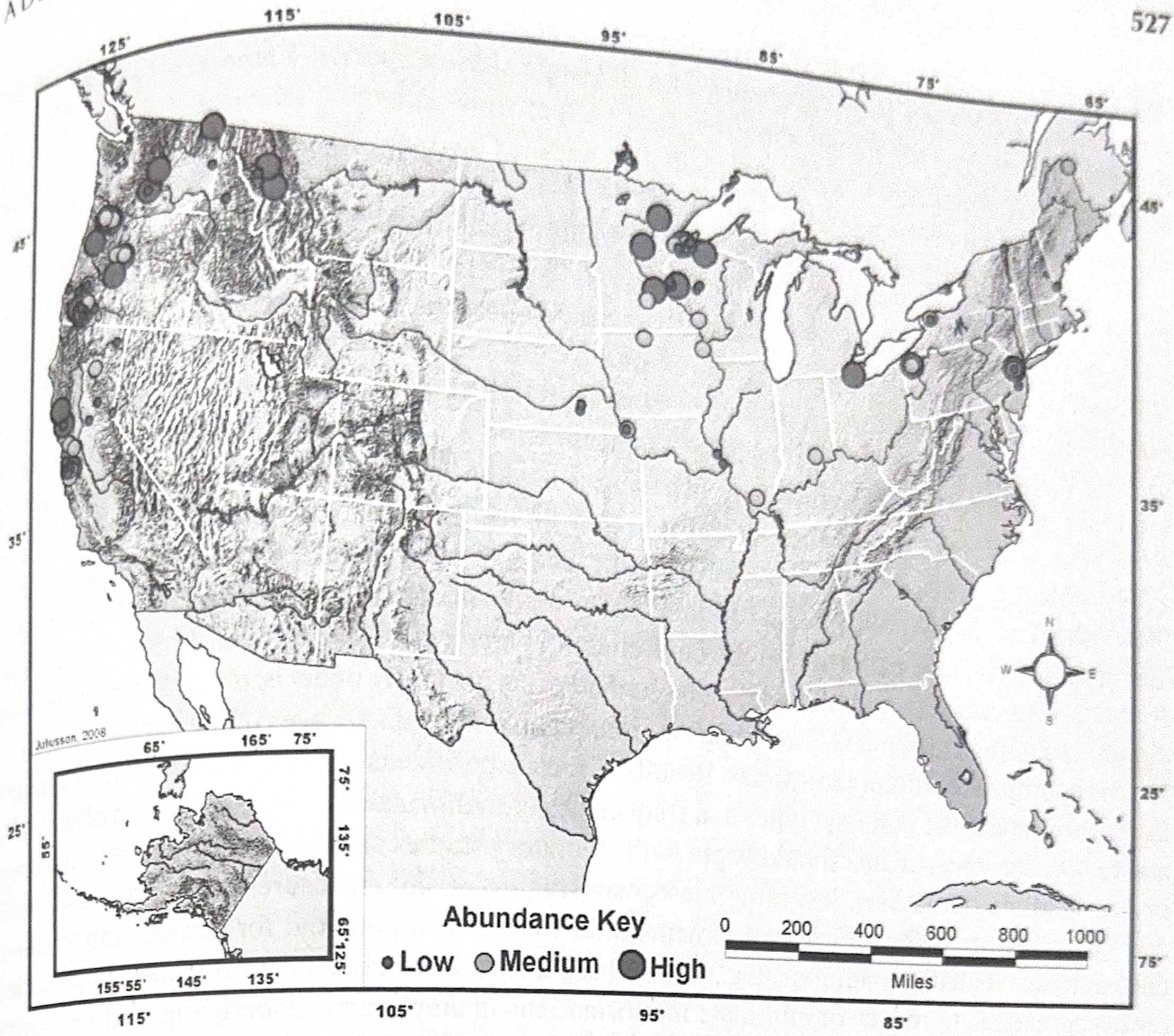


FIGURE 16.4 Geographic distribution of *Ribeiroia* from amphibians. Data represent a compilation of samples from US National Wildlife Refuges and additional sampling on private lands (sites that did not support *Ribeiroia* not depicted here). The size of each circle reflects the average infection abundance recorded in the sample (usually determined from a sample of 10 amphibians). In total, 16 amphibian species from 107 sites distributed across 20 states are included (1999 to 2007). Abundance values as follows: low (1 to 10 metacercariae per amphibian), medium (11 to 30 metacercariae), and high (31 to 135 metacercariae). Infection intensity for individual frogs ranged from 1 to 960. (Reprinted from Johnson and Mckenzie (2008), Effects of environmental change on helminth infections in amphibians: exploring the emergence of *Rebeiroia* and *Echinostoma* infections in North America. Chapter 11 (pp. 249–280) in fried, B. and R. Toledo, *The Biology of Echinostomes, from the Molecule to the Community*. Springer.)

harmful contaminants in the environment, which has kept the issue in the media spotlight. A careful review of the existing scientific literature reveals that malformations are not new to amphibians, and indeed have been recorded for over 300 years (see Ouellet 2000). However, emerging evidence suggests that, at least in some regions of the country, the prevalence of abnormal individuals is substantially greater than the expected baseline levels and may be increasing. A general lack of historical baseline data has often made interpretation of current patterns difficult.

Substantial progress has been made over the last decade in understanding amphibian malformations. Perhaps foremost among these advances is the growing recognition that multiple causes are involved, and that such causes may vary among wetlands and years but have a high potential for interaction. Nevertheless, it is imperative to recognize that simply because an agent can produce abnormalities in laboratory experiments does not mean that it actually is causing abnormalities in nature. Every agent proposed has the capacity to induce abnormalities, but without contextual field data on the concentration, intensity, or abundance of that agent in the environment, the significance of the agent's contribution to abnormalities in wild amphibian populations is difficult to determine. Because amphibian abnormalities are a series of phenomena, pursuit of a single "smoking gun" is

likely to hinder rather than help the investigation of cause-effect relationships, as it could prevent the recognition of different proximate causes for disparate sites or species. Large-scale and long-term regional surveys for amphibian abnormalities, such as those currently under way by the USFWS, are essential, particularly when accompanied by detailed data on the prevalence, composition, and extent of observed abnormalities.

Continued investigation of amphibian abnormalities demands an integrative approach, combining laboratory studies, field surveys, and large-scale experiments. Collaboration among scientists in varied fields — especially ecology, herpetology, developmental biology, and aquatic toxicology — is a necessary element in this approach. We emphasize the need for an ecologically relevant approach that follows a modified version of Koch's postulates, which are used to link an agent of disease to the pathology it causes by 1) isolating the agent from a diseased individual, 2) culturing it in vitro, and then 3) inducing illness by exposing a healthy individual to the agent. However, the narrow focus of these postulates requires that they be modified to a broader set of circumstances, as has been proposed in several other scientific fields (e.g., Glasgow et al. 2001; Grimes 2006; Plowright et al. 2008). With respect to amphibian malformations, field surveys should be used to identify malformation patterns and the environmental factors that correlate with their occurrence. Hypothesized causal factors should then be studied experimentally under ecologically relevant conditions, including experiments involving environmentally relevant dosages of the candidate agent(s) and the appropriate amphibian species. Results of such experiments can reveal whether the proposed factors are capable of inducing types and frequencies of malformations similar to those observed in nature. Ideally, experiments should begin with laboratory studies and develop into larger-scale, replicated field manipulations, including mesocosm studies, in situ enclosures/exclosures, and whole ecosystem manipulations. Field experiments offer the greatest potential for demonstrating causal control under realistic conditions (e.g., Vredenburg 2004), and may be instrumental in developing control strategies to reduce or eliminate malformations in amphibians undergoing declines. Given the potential ethical dilemmas intrinsic to adding teratogenic agents to field experiments or entire ecosystems, however, we advocate removal/elimination experiments, but acknowledge that this will be easier for some factors than others.

Finally, recognizing the potential for interactions among factors and the importance of indirect causes, research should return to the field to examine what underlying factors control or mediate the concentrations of a factor that is known to cause malformations. While this begins to deviate from Koch's postulates and the principle of parsimony, it is well founded upon the pillars of epidemiology (e.g., Lesser et al. 2007). Identifying the proximate cause of a disease (such as amphibian malformations) is only the first step; almost all emerging diseases involve environmental co-factors that influence the levels of pathology. Examples include introduced species, pollution, changes in community structure, land use change, and climate shifts (e.g., Ostfeld et al. 2008). This latter step is therefore essential in understanding amphibian malformations and mitigating or reversing their impacts on already declining amphibian populations. Thus far, none of the proposed causes of malformations have fully completed this series of causal demonstrations, and we recognize that the formula outlined here will not apply perfectly in all cases. We nevertheless suggest that it provides a useful guideline from which to develop an adaptive approach with the same general goals (Plowright et al. 2008).

Whatever causative agents are responsible, the study of amphibian abnormalities may aid the ongoing investigation of amphibian decline (e.g., Houlahan et al. 2000; Stuart et al. 2004). Amphibians have received international attention and funding as a result of abnormalities and disease, and broad-scale surveys have been initiated in many affected regions. Baseline data on the naturally occurring abnormality rates will help us to better understand the background fluctuations of amphibian population dynamics. Information on the role of pathogens and parasites in amphibians, not to mention increased attention to water quality and methods of assaying potentially harmful agents, is being generated in the ongoing study of amphibian abnormalities.

16.6 EPILOGUE

Today, more than 13 years after the original reports from this site elevated malformed frogs to national and international attention, the Ney Pond in Minnesota has been transformed into a nature center devoted to environmental education. The director of the center is one of the former students from the middle school class that first stumbled upon the deformed frogs in 1995. While *Ribeiroia* infection has been consistently documented in frogs from the pond, malformation levels have varied dramatically over the years, only rarely attaining the high frequency seen in 1995 (Vandenlangenberg et al. 2003; Johnson and Sutherland unpublished). In many respects, the patterns at this wetland illustrate both the progress we have made and the many questions that still surround this issue. Undoubtedly, parasite infection explains some fraction of the malformations observed at Ney, but how much remains unknown. Parasite infection is only part of the story. The ultimate factors that control variation in abnormalities among wetlands and among years are still at large, as are the factors that caused the "outbreak" of malformations at Ney in the 1990s. These same questions can be posed for sites that do not support *Ribeiroia*, often with even fewer answers, emphasizing the complexity of interactions among biotic and abiotic factors in controlling amphibian malformations.

The continued study of this issue requires more large-scale and long-term studies that not only document the location and frequency of abnormalities, but also examine other factors that may influence the number or type of abnormalities, such as hydrologic period (e.g., drought or excess precipitation), temperature, changes in landscape (e.g., development, agriculture), introduced predators (e.g., fish), water quality, and disease. Standard operating procedures that address collection techniques, frequency of sampling, shipping and handling of animals, and disinfecting equipment are required to limit or avoid detrimental impacts to amphibians and their habitats. Even today, variation in how abnormalities and "hot spots" are classified affects our ability to interpret large-scale patterns. Establishing standardized protocols and data sheets will help ensure that high-quality, objective data are gathered and analyzed to reduce subjectivity and variability. Importantly, however, the collection of additional data should be conducted in tandem with efforts to reduce or prevent the deleterious effects of malformations on amphibian populations. While no study has compellingly demonstrated a link between malformation and population losses in amphibians, growing evidence indicates that malformations occur alongside elevated levels of direct and indirect mortality, which becomes especially important in amphibian populations that are already declining due to habitat loss, introduced species, pollution, and other diseases. Thus, experimental efforts that strive not only to understand cause-effect relationships but also to apply such results toward lessening the impacts of malformations on amphibians are deemed particularly valuable.

GLOSSARY: MODIFIED LIST FROM OUELLET (2000) AND JOHNSON ET AL. (2001B)

Anophthalmia: Absence of one or both eyes.
Brachydactyly: Shorter digit or digits.
Brachymelia: Shorter limb or limbs.
Cutaneous fusion (skin webbing): A layer of skin connecting 2 or more limb segments.
Ectrodactyly (oligodactyly): Absence of one or more digits (adactyly) or parts of digits.
Ectromelia: Absence of one or more limbs or parts of limbs.
Edema: Fluid-filled swelling.
Hemimelia: Absence of all or part of the distal half of a limb.
Kyphosis: Abnormal backward curvature of the spine or "humpback."
Lordosis: Abnormal forward or concave curvature of the spine.

Mandibular hypoplasia: Underdeveloped mandible.
Micromelia: Shortened limb or parts of limb.
Microphthalmia: Eye smaller than normal.
Polydactyly: Supernumerary digit(s).
Polymelia: Supernumerary limb(s).
Polypody: A limb with 2 or more hands or feet.
Scoliosis: Abnormal lateral curvature of the spine.
Syndactyly: Fusion of 2 or more digits.
Synmelia: Fusion of a limb or parts of a limb to a body part.
Taumelia (bony triangle): Long bone bent at right angles to itself, often forming a triangle or pyramid.

REFERENCES

Alvarez R, Honrubia MP, Herráez MP. 1995. Skeletal malformations induced by the insecticides ZZ-Aphox® and Folidol® during larval development of *Rana perezi*. Arch Environ Contam Toxicol 28:349–356.

Ankley GT, Degitz SJ, Diamond SA, Tietge JE. 2004. Assessment of environmental stressors potentially responsible for malformations in North American anuran amphibians. Ecotoxicol Environ Saf 58:7–16.

Ankley GT, Diamond SA, Tietge JE, Holcolmbe GW, Jensen KM, Defoe DL, Peterson R. 2002. Assessment of the risk of solar ultraviolet radiation to amphibians: dose-dependent induction of hindlimb malformations in the northern leopard frog (*Rana pipiens*). Environ Sci Technol 36:2853–2858.

Ankley GT, Tietge JE, DeFoe DL, Jensen KM, Holcombe GW, Durhan EJ, Diamond SA. 1998. Effect of ultraviolet light and methoprene on survival and development of *Rana pipiens*. Environ Toxicol Chem 17:2530–2542.

Ankley GT, Tietge JE, Holdcombe GW, DeFoe DL, Diamond SA, Jensen KM, Degitz SJ. 2000. Effects of laboratory ultraviolet radiation and natural sunlight on survival and development of *Rana pipiens*. Can J Zool 78:1092–1100.

Bacon JP, Linzey DW, Rogers RL, Fort DJ 2006. Deformities in cane toad (*Bufo marinus*) populations in Bermuda. Part I. Frequencies and distribution of abnormalities. Appl Herpetol 3:39–65.

Banta BH. 1966. A six-legged anuran from California. Wasman J Biol 24:67–69.

Belden LK, Kiesecker JM. 2005. Glucocorticosteroid hormone treatment of larval tree frogs increases infection by *Alaria* sp. *trematode cercariae*. J Parasitol 91:686–688.

Bishop CA, Struger J, Sirose LJ, Dunn L, Campbell GD. 2000. Contamination and wildlife communities in stormwater detention ponds in Guelph and the greater Toronto area, Ontario, 1997 and 1998. Part II. Contamination and biological effects of contamination. Water Qual Res J Can 35:437–474.

Bishop DW. 1947. Polydactyly in the tiger salamander. J Hered 38:290–293.

Blaustein AR, Hoffman PD, Hokit DG, Kiesecker JM, Walls SC, Hays JB. 1994. UV repair and resistance to solar UV-B in amphibian eggs: a link to population declines? Proc Natl Acad Sci USA 91:1791–1795.

Blaustein AR, Johnson PTJ. 2003. The complexity of deformed amphibians. Front Ecol Environ 1:87–94.

Blaustein AR, Kiesecker JM, Chivers DP, Anthony RG. 1997. Ambient UV-B radiation causes deformities in amphibian embryos. Proc Natl Acad Sci USA 94:13735–13737.

Bohl E. 1997a. Limb deformities of amphibian larvae in Aufsess (Upper Franconia): attempt to determine causes. Münich Contr Wast Fish River Bio 50:160–189.

Bohl E. 1997b. Erfolgskontrolle der Egeldichtereduktion im Naturteich des Fischereibeispielbetriebs in Aufseß (Oberfranken: Landkreis Bayreuth). Bericht von Diplom Biologe Roland Fischer Bayreuth, September.

Boone MD, James SM. 2005. Aquatic and terrestrial mesocosms in amphibian ecotoxicology. Appl Herpetol 2:231–257.

Bowerman J, Johnson PTJ. 2003. Timing of trematode-related malformations in Oregon spotted frogs and Pacific tree frogs. Northwestern Nat 84:142–145.

Bowerman J, Johnson PTJ, Bowerman T. in press. Sublethal predators and their injured prey: vertebrate and invertebrate predators cause severe limb abnormalities in native amphibians.

Bradford DF. 1989. Allotopic distribution of native frogs and introduced fishes in high Sierra Nevada lakes of California: implication of the negative effect of fish introduction. Copeia 1989:775–778.

Bridges CM. 2000. Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). Arch Environ Contam Toxicol 39:91–96.

Bridges CM, Little E, Gardiner D, Petty J, Huckins J. 2004. Assessing the toxicity and teratogenicity of pond water in north-central Minnesota to amphibians. Environ Sci Pollut Res 11:233–239.

Brunelli E, Bernabo I, Berg C, Lundstedt-Enkel K, Bonacci A, Tripepi S. 2009. Environmentally relevant concentrations of endosulfan impair development, metamorphosis, and behaviour in *Bufo bufo* tadpoles. Aquat Toxicol 91:135–142.

Bryant SV, Gardiner DM. 1992. Retinoic acid, local cell-cell interactions, and pattern formation in vertebrate limbs. Dev Biol 152:1–25.

Bryant SV, Gardiner DM, Muneoka K. 1987. Limb development and regeneration. Am Zool 27:675–696.

Butler EG, Blum HF. 1963. Supernumerary limbs of urodele larvae resulting from localized ultraviolet light. Dev Biol 7:218–233.

Calef GW. 1973. Natural mortality of tadpoles in a population of *Rana aurora*. Ecology 54:741–758.

Carey C, Bradford DF, Brunner JL, Collins JP, Davidson EW, Longcore JE, Ouellet M, Pessier AP, Schock DM. 2003. Biotic factors in amphibian population declines. In: Linder G, Krest SK, Sparling DW, editors, Amphibian decline: an integrated analysis of multiple stressor effects. Pensacola (FL): Society of Environmental Toxicology and Chemistry, p 194.

Cecil SG, Just JJ. 1979. Survival rate, population density and development of a naturally occurring anuran larvae (*Rana catesbeiana*). Copeia 1979:447–453.

Chandler JH, Marking LL. 1975. Toxicity of the lampricide 3 tri fluoromethyl-4-nitrophenol to selected aquatic invertebrates and frog larvae. USFWS Invest Fish Cont 62:3–7.

Chen T-H, Gross JA, Karasov WH 2006. Sublethal effects of lead on northern leopard frog (*Rana pipiens*) tadpoles. Environ Toxicol Chem 25:1383–1389.

Cohen MM. 2001. Frog decline, frog malformations, and a comparison of frog and human health. Am J Med Genet 104:101–109.

Converse KA, Mattsson J, Eaton-Poole L. 2000. Field surveys of midwestern and northeastern Fish and Wildlife Service lands for the presence of abnormal frogs and toads. J Iowa Acad Sci 107:160–167.

Cooke AS. 1981. Tadpoles as indicators of harmful levels of pollution in the field. Environ Pollut 25:123–133.

Cooper JE. 1958. Some albino reptiles and polydactylous frogs. Herpetologica 14:55–56.

Crump ML. 1983. Opportunistic cannibalism by amphibian larvae in temporary aquatic environments. Am Nat 121:281–207.

Cunningham JD. 1955. Notes on an abnormal *Rana aurora draytoni*. Herpetologica 11:149.

Daszak P, Cunningham AA, Hyatt AD. 2000. Emerging infectious diseases of wildlife threats to biodiversity and human health. Science 287:443–449.

Degitz SJ, Durhan EJ, Tietge JE, Kosian PA, Holcombe GW, Ankley GT. 2003a. Developmental toxicity of methoprene and several degradation products in *Xenopus laevis*. Aquat Toxicol 64:97–105.

Degitz SJ, Holcombe GW, Kosian PA, Tietge JE, Durhan EJ, Ankley GT. 2003b. Comparing the effects of stage and duration of retinoic acid exposure on amphibian limb development: chronic exposure results in mortality, not limb malformations. Toxicol Sci 74:139–146.

Degitz, SJ, Kosian PA, Makynen EA, Jensen KM, Ankley GT. 2000. Stage- and species-specific developmental toxicity of all-trans retinoic acid in four native North American ranids and *Xenopus laevis*. Toxicol Sci 57:264–274.

de Superville D. 1740. Some reflections of generation, and on monsters, with a description of some particular monsters. Phil Trans R Soc London 41:294–307.

Dial NA, Bauer CA. 1984. Teratogenic and lethal effects of paraquat on developing frog embryos (*Rana pipiens*). Bull Environ Contam Toxicol 33:592–597.

Diamond SA, Peterson GS, Tietge JE, Ankley GT. 2002. Assessment of the risk of solar ultraviolet radiation to amphibians. Prediction of impacts in selected northern Midwestern wetlands. Environ Sci Technol 36:2866–2874.

Droin A, Fischberg M. 1980. Abnormal limbs (abl), a recessive mutation affecting the tadpoles of *Xenopus l. laevis*. Experientia 36:1286–1288.

Dubois A. 1979. Anomalies and mutations in natural populations of the Rana "esculenta" complex (Amphibia, Anura). Mitt Zool Mus Berlin 55:59–87.

Dumont J, Bantle J, LaClair JJ, Fort D, Propst T, Faulkner B, Stover E. 1997. Methoprene exposed to UV light causes developmental toxicity in *Xenopus* embryos [Abstract]. In: 18th Annual Meeting of the Society of Environmental Toxicology and Chemistry, November 16–20, 1997, p 91–92.

Eaton BR, Eaves S, Stevens C, Puchniak A, Paszkowski CA. 2004. Deformity levels in wild populations of the wood frog (*Rana sylvatica*) in three ecoregions of western Canada. J Herpetol 38: 283–287.

Eaton-Poole L, Pinkney AE, Green DE, Sutherland DR, Babbitt KJ. 2003. Investigation of frog abnormalities on National Wildlife Refuges in the Northeast US In: G. Linder G, Krest SK, Sparling DW, Little E, editors, Multiple stressor effects in relation to declining amphibian populations. Conshohocken (PA): ASTM International, p 63–78.

Fauth JE. 1990. Interactive effects of predators and early larval dynamics on the tree frog *Hyla chrysoscelis*. Ecology 71:1609–1616.
Flyaks NL, Borkin LJ. 2004. Morphological abnormalities and heavy metal concentrations in anurans of contaminated areas, eastern Ukraine. Appl Herpetol 1:229–264.
Forsyth JW. 1946. The histology of anuran limb regeneration. J Morphol 79:287–321.
Fort DJ, Rogers RL, Bacon JP. 2006a. Deformities in cane toad (*Bufo marinus*) populations in Bermuda. Part II. Progress towards characterization of chemical stressors. Appl Herpetol 3:143–172.
Fort DJ, Rogers RL, Buzzard BO, Bacon JP. 2006b. Deformities in cane toad (*Bufo marinus*) populations in Bermuda. Part III. Microcosm-based exposure pathway assessment. Appl Herpetol 3:143–172.
Fort DJ, Rogers RL, Paul RR, Miller MF, Clark C, Stover EL, Yoshioko J, Quimby F, Sower SA, Reed KL, Babbitt KA, Rolland R. 2001. Effects of pond water, sediment and sediment extract samples from New Hampshire, USA on early *Xenopus* development and metamorphosis: comparison to native species. J Appl Toxicol 21:199–209.
Fried B, Pane PL, Reddy A. 1997. Experimental infection of *Rana pipiens* tadpoles with *Echinostoma trivolvis* cercariae. Parasitol Res 83:666–669.
Fry AE. 1966. Hind limb regeneration in *Rana pipiens* larvae. Copeia 1966:530–534.
Gardiner DM, Hoppe DM. 1999. Environmentally induced limb malformations in mink frogs (*Rana septentrionalis*). J Exp Zool 284:207–216.
Gardiner D, Ndayibagira A, Grün F, Blumberg B. 2003. Deformed frogs and environmental retinoids. Pure Appl Chem 75:2263–2273.
Gilbert SF. 1997. Developmental biology. Sunderland (MA): Sinauer Associates, Inc.
Gillilland MG, Muzzall PM. 2002. Amphibians, trematodes, and deformities: an overview from southern Michigan. Comp Parasitol 69:81–85.
Glaser JD. 1998. Effects of Vision (glyphosate) on progeny of wood frogs exposed in conifer plantations. MSc thesis, University of Guelph, Ontario, Canada.
Glasgow HB, Burkholder JM, Mallin MA, Deamer-Melia NJ, Reed RE. 2001. Field ecology of toxic *Pfiesteria* complex species and a conservation analysis of their role in estuarine fish kills. Environ Health Perspect 109:715–730.
Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190.
Grant KP, Licht LR. 1995. Effects of ultraviolet radiation of life-history stages of anurans from Ontario, Canada. Can J Zool 73:2292–2301.
Gray RH. 2000. Historical occurrence of malformations in the cricket frog, *Acris crepitans*, in Illinois. Trans Illinois State Acad Sci 93:279–284.
Grimes J. 2006. Koch's postulates — then and now. Microbe 1:223–228.
Guderyahn L. 2006. Nationwide assessment of morphological abnormalities observed in amphibians collected from United States National Wildlife Refuges. CBFO-C0601. Annapolis (MD): USFWS.
Gurushankara HP, Krishnamurthy SV, Vasudev V. 2007. Morphological abnormalities in natural populations of common frogs inhabiting agroecosystems of the central Western Ghats. Appl Herpetol 4:39–45.
Harmon MA, Boehm MF, Heyman RA, Mangelsdorf DJ. 1995. Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. Proc Natl Acad Sci USA 92:6157–6160.
Harris ML, Bishop CA, McDaniel TV. 2001. Assessment of rates of deformity in wild frog populations using in situ enclosures: a case study of leopard frogs (*Rana pipiens*) in Ontario, Canada. Biomarkers 6:52–63.
Harris ML, Chora L, Bishop CA, Bogart JP. 2000. Species- and age-related differences in susceptibility to pesticide exposure for two amphibians, *Rana pipiens* and *Bufo americanus*. Bull Environ Contam Toxicol 64:263–270.
Harvell CD, Titchell CE, Ward JR, Altizer S, Dobson AP, Ostfeld RS, Samuel MD. 2002. Climate warming and disease risks for terrestrial and marine biota. Science 296:2158–2162.
Hauver RC. 1958. Studies on natural anomalies of the hind limbs of *Rana catesbeiana*. Master's thesis, Miami University, Miami, OH.
Hays JB, Blaustein AR, Kiesecker JM, Hoffman PD, Pandelova I, Coyle D, Richardson T. 1996. Developmental response of amphibians to solar and artificial UV-B sources: a comparative study. Photochem Photobiol 64:449–456.
Hebard WB, Brunson RB. 1963. Hind limb anomalies of a western Montana population of the Pacific tree frog, *Hyla regilla*. Copeia 3:570–572.
Helgen JC, McKinnell RG, Gernes MC. 1998. Investigation of malformed northern leopard frogs in Minnesota. In: Lanoo MJ, editor, Status and conservation of Midwestern Amphibians. Iowa City (IA): University of Iowa, p 288–297.

Henrick CA, Ko J, Nguyen J, Burleson J, Lindahl G, Van Gundy D, Edge JM. 2002. Investigation of the relationship between s-methoprene and deformities in anurans. J Am Mosquito Cont Assoc 18:214–221.

Honrubia MP, Herraez MP, Alvarez R. 1993. The carbamate insecticide ZZ-Aphox® induced structural changes of gills, liver, gall-bladder, heart, and notochord of *Rana perezi* tadpoles. Arch Environ Contam Toxicol 25:184–191.

Hopkins WA, Congdon J, Ray JK. 2000. Incidence and impact of axial malformations in larval bullfrogs (*Rana catesbeiana*) developing in sites polluted by a coal-burning power plant. Environ Toxicol Chem 19:862–868.

Hopkins WA, Mendonça MT, Rowe CL, Congdon JD. 1998. Elevated trace element concentrations in southern toads, *Bufo terrestris*, exposed to coal combustion waste. Arch Environ Contam Toxicol 35:325–329.

Hoppe DM. 2000. History of Minnesota frog abnormalities: do recent findings represent a new phenomenon? In: Kaiser H, Casper GS, Bernstein N, editors, Investigating amphibian declines: proceedings of the 1998 declining amphibians conference. J Iowa Acad Sci 107:86–89.

Hoppe DM. 2005. Malformed frogs in Minnesota: history and interspecific differences. In: Lannoo MJ, editor, Amphibian declines: the conservation status of United States species. Berkeley (CA): University of California Press, p 75–87.

Houck WJ, Henderson C. 1953. A multiple appendage anomaly in the tadpole stage of *Rana catesbeiana*. Herpetologica 9:76–77.

Houlahan JE, Findlay CS, Schmidt BR, Meyer AH, Kuzmin SL. 2000. Quantitative evidence for global amphibian population declines. Nature 404:752–755.

Jennings WB, Bradford DF, Johnson DF. 1992. Dependence of the garter snake *Thamnophis elegans* on amphibians in the Sierra Nevada of California. J Herpetol 26:503–505.

Johnson PTJ, Chase JM. 2004. Parasites in the food web: linking amphibian malformations and aquatic eutrophication. Ecol Lett 7:521–526.

Johnson PTJ, Chase JM, Dosch KL, Hartson RB, Gross JA, Larson DJ, Sutherland DR, Carpenter SR. 2007. Aquatic eutrophication promotes pathogenic infection in amphibians. Proc Natl Acad Sci USA 104:15781–15786.

Johnson PTJ, Hartson RB. 2009. All hosts are not equal: explaining differential patterns of malformations in an amphibian community. J Anim Ecol 78:191–201

Johnson PTJ, Lunde KB. 2005. Parasite infection and limb malformations: A growing problem in amphibian conservation. In: Lannoo MJ, editor, Amphibian declines: the conservation status of United States species. Berkeley (CA): University of California Press, p 124–138.

Johnson PTJ, Lunde KB, Haight RW, Bowerman J, Blaustein AR. 2001a. *Ribeiroia ondatrae* (Trematoda: Digenea) infection induces severe limb malformations in western toads (*Bufo boreas*). Can J Zool 79:370–379.

Johnson PTJ, Lunde KB, Ritchie EG, Launer AE. 1999. The effect of trematode infection on amphibian limb development and survivorship. Science 284:802–804.

Johnson PTJ, Lunde KB, Ritchie EG, Reaser JK, Launer AE. 2001b. Morphological abnormality patterns in a California amphibian community. Herpetologica 57:336–352.

Johnson PTJ, Lunde KB, Thurman EM, Ritchie EG, Wray SN, Sutherland DR, Kapfer JM, Frest TJ, Bowerman J, Blaustein AR. 2002. Parasite (*Ribeiroia ondatrae*) infection linked to amphibian malformations in the western United States. Ecol Monogr 72:151–168.

Johnson PTJ, Lunde KB, Zelmer DA, Werner JK. 2003. Limb deformities as an emerging parasitic disease in amphibians: evidence from museum specimens and resurvey data. Conserv Biol 17:1724–1737.

Johnson PTJ, Preu ER, Sutherland DR, Romansic J, Han B, Blaustein AR. 2006. Adding infection to injury: synergistic effects of predation and parasitism on salamander limb malformations. Ecology 87:2227–2235.

Johnson PTJ, Sutherland DR, Kinsella JM, Lunde KB. 2004. Review of the trematode genus *Ribeiroia* (Psilostomidae): ecology, life history, and pathogenesis with special emphasis on the amphibian malformation problem. Adv Parasitol 57:191–253.

Kaiser J. 1997. Ecology — deformed frogs leap into spotlight at health workshop. Science 278:2051–2052.

Kaiser J. 1999. Frog declines — deformities — a trematode parasite causes some frog deformities. Science 284:731.

Kerr JB, McElroy CT. 1993. Evidence for large upward trends of ultraviolet-B radiation linked to ozone depletion. Science 262:1032–1034.

Kiesecker JM. 2002. Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? Proc Natl Acad Sci USA 99:9900–9904.

Kiesecker JM, Blaustein AR. 1997. Population differences in responses of red-legged frogs (*Rana aurora*) to introduced bullfrogs. Ecology 78:1752–1760.

Koprivnikar J, Forbes MR, Baker RL. 2007. Contaminant effects on host-parasite interactions: atrazine, frogs and trematodes. Environ Toxicol Chem 26:2166–2170.
Kupferberg SJ, Catenazzi A, Lunde K, Palen WJ. 2009. Parasitic copepod (*Lernaea cyprinacea*) outbreaks in foothill yellow-legged frogs (*Rana bovlii*) linked to unusually warm summers and amphibian malformations in northern California. Copeia 2009(3): 529–537.
Kurabuchi S, Inoue S. 1982. Limb regenerative capacity of four species of Japanese frogs of the families Hylidae and Ranidae. J Morphol 173:129–135.
Lannoo MJ. 2003. Conclusions drawn from the malformity and disease session, Midwest Declining Amphibian Conference, 1998. J Iowa Acad Sci 107:212–216.
Lannoo MJ. 2008. Malformed frogs. The collapse of aquatic ecosystems. Berkeley (CA): University of California Press.
Lannoo MJ, Sutherland DR, Jones P, Rosenberry D, Klaver RW, Hoppe DM, Johnson PTJ, Lunde KB, Facemire C, Kapfer JM. 2003. Multiple causes for the malformed frog phenomenon. In: Linder G, Little E, Krest S, Sparling D, editors, Multiple stressor effects in relation to declining amphibian populations. ATSM STP. West Conshoshocken (PA): ASTM International, p 1443.
Lesser MP, Bythell JC, Gates RD, Johnstone RW, Hoegh-Guldberg O. 2007. Are infectious diseases really killing corals? Alternative interpretations of the experimental and ecological data. J Exp Mar Biol Ecol 346:36–44.
Levey R. 2003. Investigations into the causes of amphibian malformations in the Lake Champlain Basin of New England. Waterbury (VT): Vermont Department of Environmental Conservation.
Licht LE. 1974. Survival of embryos, tadpoles, and adults of the frogs *Rana aurora aurora* and *Rana pretiosa pretiosa* in southwestern British Columbia. Can J Zool 52:613–627.
Loeffler IK, Stocum DL, Fallon JF, Meteyer CU. 2001. Leaping lopsided: a review of the current hypotheses regarding etiologies of limb malformations in frogs. Anat Rec 265: 228–245.
Lopez TJ, Maxson L. 1990. *Rana catesbeiana* polymely. Herpetol Rev 21:90.
Maden M. 1993. The homeotic transformation of tails into limbs in *Rana temporaria* by retinoids. Dev Biol 159:379–391.
Malacinski GM, Allis CD, Chung HM. 1974. Correction of developmental abnormalities resulting from localized UV irradiation of an amphibian egg. J Exp Zool 189:249–254.
Marchal-Segault D, Ramade F. 1981. The effects of lindane, an insecticide, on hatching postembryonic development of *Xenopus laevis* (Dauding) anuran amphibian. Environ Res 24:250–258.
Martof B. 1956. Factors influencing size and composition of populations of *Rana clamitans*. Am Mid Nat 56:224–245.
McCallum ML, Trauth SE. 2003. A forty-three year museum study of northern cricket frog (*Acris crepitans*) abnormalities in Arkansas: upward trends and distributions. J Wildl Dis 39:522–528.
McKenzie R, Connor B, Bodeker G. 1999. Increased summertime UV radiation in New Zealand in response to ozone loss. Science 285: 1709–1711.
Merrell J. 1969. Natural selection in the leopard frog population. J Minn Acad Sci 35:86–89.
Meteyer CU, Loeffler IK, Fallon JF, Converse KA, Green E, Helgen JC, Kersten S, Levey R, Eaton-Poole L, Burkart JG. 2000. Hind limb malformations in free-living northern leopard frogs (*Rana pipiens*) from Maine, Minnesota, and Vermont suggest multiple etiologies. Teratology 62:151–171.
Murphy TD. 1965. High incidence of two parasitic infestations and two morphological abnormalities in a population of the frog, *Rana palustris* Le Conte. Am Mid Nat 74:233–239.
[NARCAM] North American Reporting Center for Amphibian Malformations. 2008. National Biological Information Infrastructure. Available from: http://frogweb.nbii.gov/narcam/. Accessed August 27, 2008.
Ostfeld RS, Keesing F, Eviner VT. 2008. Infectious disease ecology: effects of ecosystems on disease and of disease on ecosystems. Princeton (NJ): Princeton University Press.
Ouellet M. 2000. Amphibian deformities: current state of knowledge. In: Sparling DW, Linder G, Bishop CA, editors, Ecotoxicology of amphibians and reptiles. Pensacola (FL): Society of Environmental Toxicology and Chemistry, p 617–661.
Ouellet M, Bonin J, Rodrigue J, DesGranges J, Lair S. 1997. Hindlimb deformities (ectromelia, ectrodactyly) in free living anurans from agricultural habitats. J Wildl Dis 33:95–104.
Ovaska K, Davis TM, Flamarique IN. 1997. Hatching success and larval survival of the frogs *Hyla regilla* and *Rana aurora* under ambient and artificially enhanced solar ultraviolet radiation. Can J Zool 72:1081–1088.
Palen WJ, Schindler DE, Adams MJ, Pearl CA, Bury RB, Diamond SA. 2002. Optical characteristics of natural waters protect amphibians from UV-B in the U.S. Pacific Northwest. Ecology 83:2951–2957.
Pawar KR, Ghate HV, Katdare M. 1983. Effect of malathion on embryonic development of the frog *Microhyla ornata*. Bull Environ Contam Toxicol 31:170–176.

Peterson GS, Johnson LB, Axler RP, Diamond SA. 2002. Assessment of the risk of solar ultraviolet radiation to amphibians. II. In situ characterization of exposure in amphibian habitats. Environ Sci Technol 36:2859–2865.

Piha HP, Pekkonen M, Merilä J. 2006. Morphological abnormalities in amphibians in agricultural habitats: a case study of the common frog *Rana temporaria*. Copeia 2006:810–817.

Pinkney AE, Eaton-Poole L, LaFiandra EM, Babbitt KJ, Bridges-Britton CM, Little EE, Cranor WL. 2006. Investigation of contaminant effects on frog development at Great Bay National Wildlife Refuge, Newington, New Hampshire. USFWS CBFO-C0602 and RY2006-NEFO-EC-01. Annapolis (MD): US Fish and Wildlife Service.

Plowright et al. (2008). Causal inference in disease ecology: investigating ecological drivers of disease emergence. Frontiers in Ecology and the Environment 6: 420–429.

Rajakaruna RS, Piyatissa PMJR, Jayawardena UA, Navaratne AN, Amerasinghe PH. 2008. Trematode infection induced malformations in the common hourglass treefrogs. J Zool 275:89–95.

Reeves MK, Dolph CL, Zimmer H, Tjeerdema RS, Trust KA. 2008. Road proximity increases risk of skeletal abnormalities in wood frogs from National Wildlife Refuges in Alaska. Environ Health Perspect 116:1009–1015.

Relyea RA. 2005. The lethal impacts of Roundup and predatory stress on six species of North American tadpoles. Arch Environ Contam Toxicol 48:351–357.

Relyea RA, Hoverman J. 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. Ecol Lett 9:1157–1171.

Reynolds TD, Stephens TD. 1984. Multiple ectopic limbs in a wild population of *Hyla regilla*. Great Basin Nat 44:166–169.

Rohr JR, Schotthoefer AM, Raffle TR, Carrick HJ, Halstead N, Hoverman JT, Johnson CM, Johnson LB, Lieske C, Piwoni MD, Schoff PK, Beasley VR. 2008. Agrochemicals increase trematode infections in a declining amphibian species. Nature 455:1235–1239.

Rosenshield ML, Jofre MB, Karasov WH. 1999. Effects of polychlorinated biphenyl 126 on green frog (*Rana clamitans*) and leopard frog (*Rana pipiens*) hatching success, development, and metamorphosis. Environ Toxicol Chem 18:2478–2486.

Rostand J, Darré P. 1969. Action tératogène des déjections de certains poisons sur les larves de *Rana esculenta*. C R Soc Biol 163:2033–2034.

Ruth FS. 1961. Seven-legged bullfrog, *Rana catesbeiana*. Turtox News 39:232.

Schmidt CW. 1997. Amphibian deformities continue to puzzle researchers. Environ Sci Technol 31:324–326.

Schoff PK, Johnson CM, Schotthoefer AM, Murphy JE, Lieske C, Cole RA, Johnson LB, Beasley VR. 2003. Prevalence of skeletal and eye malformations in frogs from north-central United States: estimations based on collections from randomly selected sites. J Wildl Dis 39:510–521.

Schotthoefer AM, Cole RA, Beasley VR. 2003. Relationship of tadpole stage to location of echinostome cercariae encystment and the consequences for tadpole survival. J Parasitol 89:475–482.

Schotthoefer AM, Labak KM, Beasley VR. 2007. *Ribeiroia ondatrae* cercariae are consumed by aquatic invertebrate predators. J Parasitol 93:1240–1243.

Schuytema GS, Nebeker AV, Griffis WL. 1994. Toxicity of Guthion and Guthion 2S to *Xenopus laevis* embryos. Arch Environ Contam Toxicol 27:250–255.

Schuytema GS, Nebeker AV, Griffis WL, Wilson KN. 1991. Teratogenesis, toxicity, and bioconcentration in frogs exposed to Dieldrin. Arch Environ Contam Toxicol 21:332–350.

Sessions SK, Ruth SB. 1990. Explanation for naturally-occurring supernumerary limbs in amphibians. J Exp Zool 254:38–47.

Skelly DK, Bolden SR, Freidenburg LK, Freidenfelds NA, Levey R. 2007. *Ribeiroia* infection is not responsible for Vermont amphibian deformities. EcoHealth 4:156–163.

Souder W. 2000. A plague of frogs. New York: Hyperion Press.

Sparling DW. 2000. Effects of Altosid® and Abate 4E® on deformities and survival in southern leopard frogs under semi-natural conditions. J Iowa Acad Sci 107:90–91.

Sparling DW, Krest S, Ortiz-Santaliestra M. 2006. Effects of lead-contaminated sediment on *Rana spheno-cephala* tadpoles. Arch Environ Contam Toxicol 51:458–466.

Stopper GF, Hecker L, Franssen RA, Sessions SK. 2002. How trematodes cause limb deformities in amphibians. J Exp Zool 294:252–263.

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigue ASL, Fischman DL, Waller RW. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Surlève-Bazeille JE, Cambar R. 1969. Nouvelles recherches sur les modalités de transmission de l'anomalie P chez la grenouille verte (*Rana esculenta* L.) atteintes de l'anomalie P. C R Soc Biol 163:2034–2037.

Surlève-Bazeille JE, Cambar R, Calas A. 1969. Premières observations histologiques sur les members des larves de grenouille verte (*Rana esculenta* L.) atteints de l'anomalie P. C R Acad Sci 269:2240–2242.

Sutherland DR. 2005. Parasites of North American frogs. In: Lannoo MJ, editor, Amphibian declines: the conservation status of United States species. Berkeley (CA): University of California Press, p 109–123.

Taylor B, Skelly D, Demarchis LK, Slade MD, Galusha D, Rabinowitz PM. 2005. Proximity to pollution sources and risk of amphibian limb malformations. Environ Health Perspect 113:1497–1501.

Thieltges DW, Jensen KT, Poulin R. 2008. The role of biotic factors in the transmission of free-living endohelminth stages. Parasitology 135:407–426.

Thiemann GW, Wassersug RJ. 2000. Patterns and consequences of behavioural responses to predators and parasites in *Rana* tadpoles. Biol J Linn Soc 71:513–528.

Thul JE, Forrester DJ, Abercrombie CL. 1985. Ecology of parasitic helminths of wood ducks *Aix sponsa*, in the Atlantic flyway. Proc Helminol Soc Wash 52:297–310.

Turley SD, Eaton-Poole L, Pinkney AE, Osborn MA, Burton DT. 2003. Evaluation of the potential impact of water and sediment from National Wildlife Refuge sites using a modified Frog Embryo Teratogenesis Assay–Xenopus (FETAX). In: Linder G, Krest SK, Sparling DW, Little E, editors, Multiple stressor effects in relation to declining amphibian populations. West Conshohocken (PA): American Society for Testing and Materials (ASTM), p 79–94.

[USFWS] US Fish and Wildlife Service. 2009. Assessment of frog abnormalities on U.S. National Wildlife Refuges (2000–2005): report to Congress. Arlington (VA): USFWS, Division of Environmental Quality.

Vallisneri A. 1706. Galleria di Minerva. Vol. V:285. Venice (IT). (As cited by Taruffi 1885.)

Vandenlangenberg SM, Canfield JT, Magner JA. 2003. A regional survey of malformed frogs in Minnesota (USA). Environ Monit Assess 82:45–61.

Van der Schalie WH, Gardner HS, Bantle JA, De Rosa CT, Finch RA, Reif JF, Reuter RH, Backer LC, Burger J, Folmar LC, Stokes WS. 1999. Animals as sentinels of human health hazards of environmental chemicals. Environ Health Perspect 107:309–315.

Van Valen L. 1974. A natural model for the origin of some higher taxa. J Herpetol 8:109–121.

Veith M, Viertel B. 1993. Veršnderungen an den extremitšten von Larven und Jungtieren der erdkršte (Bufo bufo): Analyse mšglicher Ursachen. Salamandra 29:184–199.

Viertel B, Veith M. 1992. Predation by leeches and regeneration, a factor in larval development of *Bufo bufo* (L.). Proc Sixth Ord Gen Meet S E H Budapest 1991:479–484.

Volpe EP. 1977. Understanding evolution. 3rd ed. Dubuque (IA): Wm. C. Brown Co. Publishers.

Vredenburg VT. 2004. Reversing introduced species effects: experimental removal of introduced fish leads to rapid recovery of a declining frog. Proc Natl Acad Sci USA 101:7646–7650.

Walls SC, Beatty JJ, Tissot BN, Hokit DG, Blaustein AR. 1993. Morphological variation and cannibalism in a larval salamander (*Ambystoma macrodactylum columbianum*). Can J Zool 71:1543–1551.

Wildy EL, Chivers DP, Kiesecker JM, Blaustein AR. 2001. The effects of food level and conspecific density on biting and cannibalism in larval long-toed salamanders, *Ambystoma macrodactylum*. Oecologia 128:202–209.

Williams RN, Bos DH, Gopurenko D, DeWoody JA. 2008. Amphibian malformations and inbreeding. Biol Lett 4:549–552.

Woodward BD. 1983. Predator-prey interactions and breeding-pond use of temporary-pond species in a desert community. Ecology 64:1549–1555.

Worrest RC, Kimeldorf DJ. 1976. Distortions in amphibian development induced by ultraviolet-B enhancement (290–315 nm) of a simulated solar spectrum. Photochem Photobiol 24:377–382.