The complexity of deformed amphibians

Andrew R Blaustein[1] and Pieter TJ Johnson[2]

Many amphibian populations have disappeared or are in decline throughout the world. In addition, more than 60 different species of amphibians with severe abnormalities have been found in the US and several other countries. These complex, perhaps interrelated phenomena are associated with important current challenges in conservation biology. Although intense research, beginning in the early 1990s, has led to a better understanding of why amphibian populations are declining, there is still a basic lack of knowledge about the causes and implications of amphibian deformities.

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Global biodiversity loss and the emergence of infectious diseases are two of the most pressing environmental concerns, and the underlying causes are both complex and intertwined. Some estimates suggest that the current rate of extinction is greater than any known in the last 100 000 years (Wilson 1992; Eldredge 1998). Pathogens are one important factor that can threaten biodiversity by accelerating population declines, leading to extinctions (Daszak *et al.* 1999, 2000; Harvell *et al.* 2002). The past several decades have witnessed an unprecedented number of emerging and reemerging diseases, often with serious repercussions for humans and wildlife (Daszak *et al.* 2000; Harvell *et al.* 2002).

Two specific phenomena associated with the biodiversity crisis and the increase in infectious diseases are the global decline of amphibian populations and the appearance of large numbers of deformed amphibians. According to some recent estimates, more than 500 populations of frogs, toads, and salamanders are in decline or at risk (Alford and Richards 1999; Houlahan *et al.* 2000). Associated with some of these disappearances are reports of massive mortality and, in several populations, an increasing incidence of developmental malformations. More than 80% of the individuals in some populations exhibit severe deformities, including missing limbs or numerous extra legs (Johnson *et al.* 2002; Figure 1).

These widespread reports signal an emerging problem in conservation biology. Although there have been several recent reviews concerning decreases in amphibian populations (Alford and Richards 1999; Houlahan *et al.* 2000; Blaustein and Kiesecker 2002), there have been few reviews of the current state of knowledge concerning amphibian deformities. In this paper, we review the causes, implications, and significance of these abnormalities.

In a nutshell:

- Two critical issues facing scientists are global biodiversity loss and the emergence of infectious diseases
- Associated with these problems are the global decline of amphibian populations and the increasing frequency of deformations among these species
- Both appear to be a result of multiple causes, all related to human-induced environmental damage, including contamination, increasing ultraviolet radiation, and parasitic infection
- A coordinated interdisciplinary approach is necessary to tackle this problem

Bioindicators of environmental stress

Amphibians are considered by many biologists to be excellent "bioindicators" of environmental health (Blaustein 1994; Blaustein and Wake 1995). As a result, this class has been the subject of many recent studies and the focus of intense media attention (Souder 2000). Amphibians have permeable skin, free of scales, hair, or feathers, and shell-less eggs whose contents are directly exposed to the environment and readily absorb substances. They are also ectotherms, and the complex life cycles of many species expose them to both aquatic and terrestrial environmental hazards. Taken together, these features make them especially sensitive to changes in temperature, precipitation, and ultraviolet radiation. Because many species do not venture far from where they were hatched, amphibians also function as monitors of local conditions (Blaustein *et al.* 1994). Falling numbers, coupled with an increasing incidence of deformities, could be a warning of severe environmental degradation.

Amphibian deformities

Frogs, toads, and salamanders with extra limbs have generated scientific curiosity for centuries (Van Valen 1974; Ouellet 2000). The recent increase in this phenomenon, however, is notable for its severity. While a small number of abnormalities from mutation, developmental errors, and trauma can be expected in any amphibian population, this proportion is typically under 5%, and most

[1]*Department of Zoology, Oregon State University, 3029 Cordley Hall, Corvallis, OR 97331-2914 (blaustea@bcc.orst.edu);* [2]*Center for Limnology, University of Wisconsin, 680 North Park St, Madison, WI 53706-1492*

Courtesy of Steven Holt.

Figure 1. *Pacific treefrog (*Hyla regilla*) with two extra hind limbs.*

often involves only missing digits or parts of a limb. Whereas most historical articles describe one or possibly two affected frogs in a population, contemporary observations document high frequencies of severe deformations (15-90%), which often afflict several species at a single site (Ouellet *et al.* 1997; Helgen *et al.* 1998; Johnson *et al.* 2002). A grotesque spectrum of abnormalities has been recorded, including missing and partially missing limbs, multiple extra limbs and digits, and incomplete limb formation. Misshapen eyes and tails, skin lesions, and whole-body deformities have also been reported.

Documented cases involving high frequencies of deformed individuals in a single population are historically uncommon (Johnson *et al.* unpublished). Since the mid 1990s, however, at least 60 different species have been found to be affected in 46 US states and parts of Canada, Japan, and several European nations. The most severely affected areas include the western US, the Midwest, and southeastern Canada. Growing evidence suggests that the problem is becoming more common (Stocum 2000), but increased awareness and surveillance must also be considered. In several populations, more than half of the individuals had extra legs or were missing a limb (Figures 1 and 2; Johnson *et al.* 2002). Hoppe (unpublished) compared the incidence and types of abnormalities in Minnesota frog populations from 1996 to 1997 with those found in frogs from the same sites between 1958 and 1963. The results indicated that deformities have become more severe, more widespread, and more abundant (a sixfold increase).

The deformity issue is complex because it deals with water quality, physiology, development, anatomy, ecology, and potential effects on human health, and therefore it attracts the attention of scientists from a wide array of disciplines and government agencies. The possible causes of amphibian deformities fall into three broad categories: increasing ultraviolet (UV) radiation (Ankley *et al.* 2000, 2002), chemical contamination (Gardiner and Hoppe 1999; Burkhart *et al.* 1998), and parasitic infection (Sessions and Ruth 1990; Johnson *et al.* 1999). All three hypotheses have support, yet each has its problems as well.

UV-B radiation

Throughout evolutionary history, UV radiation has been a ubiquitous stressor on living organisms (Cockell 2001). Natural events such as comet and asteroid impacts, volcanic activity, supernova explosions, and solar flares can cause temporary but large-scale ozone depletion, with accompanying increases in UV radiation (Cockell and Blaustein 2000; Cockell 2001). However, human-induced production of chlorofluorocarbons (CFCs) and other chemicals continuously deplete stratospheric ozone, exposing plants, animals, and microorganisms to long-term, continual doses of harmful UV-B (280–315 nm) radiation. This is extremely important biologically, since it can cause mutations and cell death. In amphibians, UV-B radiation can slow growth rates, hamper the immune system, and induce many types of non-lethal damage, including malformations of the limbs, body, and eyes, as well as changes in behavior (Blaustein and Belden 2003).

In laboratory experiments, salamanders exposed to extremely high levels of UV-B radiation (orders of magnitude above current levels) developed extra limbs (Butler and Blum 1963). In field experiments, salamanders exposed to natural sunlight developed significantly more deformities of the body, tail, and eyes than salamanders that were shielded from sunlight (Blaustein *et al.* 1997). Exposure to simulated "natural" levels of UV-B radiation in the laboratory can result in curvature of the spine and abnormal skin and eye development in frogs and toads (Worrest and Kimeldorf 1976). In the wild, ambient UV-

B radiation causes severe retinal damage in basking frogs (Fite *et al.* 1998).

Because levels of UV-B radiation are increasing at the earth's surface (Kerr and McElroy 1993; Middleton *et al.* 2001), and previous research has shown that UV-B radiation can induce a variety of physical abnormalities in amphibians, it is logical to examine its role. Indeed, several recent experimental studies have shown that leopard frogs (*Rana pipiens*) exposed to UV-B radiation experienced changes in hind limb structure (Ankley *et al.* 2000, 2002).

There are several problems with blaming UV radiation for all amphibian limb deformities, however. Most important, the types of abnormalities produced in recent UV experiments (Ankley *et al.* 2000, 2002) generally do not correspond to most of those observed in the field. For example, most of the experiments resulted in bilateral limb irregularities or reductions in the number of digits. In the field, however, amphibians exhibit a wide diversity of aberrations that are limited to one side, including skin webbings and missing, twisted, or extra limbs. Dozens of laboratory and field experiments conducted in the past decade have provided no evidence that exposure to ambient levels of UV radiation causes extra legs in amphibians (reviewed in Blaustein *et al.* 1998, 2001). Furthermore, amphibian larvae and adults can limit their exposure by moving in and out of sunlight, living in muddy water, or coming out only at night. Ultraviolet radiation, therefore, seems to be an unlikely culprit for the high incidence of amphibian limb deformities found in nature.

Chemical contaminants

Perhaps the most alarming aspect of the increase in developmental problems is the possibility that contaminated water may be to blame – a situation that would have far-reaching ecological and social consequences. Numerous laboratory studies have shown that many different contaminants can kill or cause deformations in amphibians (Sparling *et al.* 2000). Deformed frogs have been found in or near sources of human drinking water, and many malformed amphibians occur in agricultural areas where insecticides and fertilizers are applied extensively (Ouellet *et al.* 1997; Hayes *et al.* 2002a, 2002b). Millions of tons of hundreds of types of pesticides and pollutants accumulate each year in areas where affected amphibians have been found, and herbicides, fungicides, heavy metals, and numerous pollutants also permeate amphibian habitats. Contaminants applied locally may be transported through the atmosphere to remote, relatively undisturbed regions, where even low levels may be harmful to these animals.

Are these products responsible for the increasing incidence of limb deformities? One major uncertainty centers on the difficulty of isolating a particular chemical, or even a group of chemicals, in nature. In the mid 1990s, several research groups focused on the potential role of retinoids, a group of compounds derived from vitamin A that can produce a suite of limb abnormalities under laboratory conditions. Retinoids can cause skeletal irregularities, incomplete or under-developed limbs, and a variety of other changes. The similarity to malformations observed recently in North American amphibian populations and those induced by experimental exposure to retinoids, led to the hypothesis that retinoids or retinoid-like compounds may play a role in amphibian limb abnormalities (Gardiner and Hoppe 1999). Initial studies focused on methoprene, a common insecticide that breaks down into a compound similar to retinoic acid under UV irradiation. However, experiments exposing amphibian larvae to methoprene in the presence and absence of UV radiation found no effects on limb development beyond those attributable to UV alone (Ankley *et al.* 1998), and no significant correlation has been found between deformed frogs and the presence of methoprene or its metabolites in the field.

Degitz *et al.* (2000) exposed larvae of African clawed frogs (*Xenopus* sp) and four North American species of ranid frogs to retinoic acid, and observed abnormalities such as limb reductions and bony triangles (long bones that appear to be bent such that the midpoint of the bone forms the peak of a triangle). However, the concentrations of retinoic acid necessary to cause these defects were lethal to amphibian embryos, leading the authors to conclude that retinoic acid was probably not the cause of these changes under field conditions. There is also very little evidence that retinoids are found in regions where

Courtesy of Daniel Sutherland.

Figure 2. *A frog infected experimentally with "Ribeiroia". The animal has been cleared and double-stained to show its severely deformed skeleton. Inset: the culprit – a "Ribeiroia" metacercariae.*

Figure 3. *Simplified life cycle of the trematode* Ribeiroia ondatrae*. The parasite reproduces asexually inside aquatic snail hosts (*Planorbella *sp), generating thousands of infectious cercariae (larvae). These burrow into the developing limb buds of amphibians and form resting cysts called metacercariae, which cause improper limb formation and are suspected to increase the odds of predation by water birds. Once inside a bird, the parasite reproduces sexually and releases eggs back into the water, where they hatch and infect aquatic snails.*

deformed amphibians have been observed.

Bony triangles, once thought to indicate retinoid exposure, have been induced through parasite infection (Johnson *et al.* 1999) and the mechanical rearrangement of developing limb cells (Stopper *et al.* 2002). Sessions *et al.* (1999) also point out that some types of malformations caused by experimental exposure to retinoids are rare in frogs caught in the field. Nevertheless, little is known about environmental retinoids or how to detect them, and more information is needed to evaluate fully their role in harming amphibians.

In a broad-scale survey in the western US, Johnson *et al.* (2002) found no connection between pesticide contamination and amphibian deformities. Pesticides cannot be completely ruled out, however. At least one insecticide, carbaryl, caused a very low incidence of missing, deformed, and extra limbs in one frog species under experimental conditions (Bridges 2000).

■ Parasites

Trematodes are parasitic flatworms with complex life cycles, typically involving two or more hosts (Figure 3). They are involved in a variety of human diseases, including schistosomiasis, a debilitating illness that afflicts about 200 million people in tropical regions. A connection between parasitic infection and amphibian limb deformities was first suggested by Sessions and Ruth (1990), who observed numerous limb abnormalities and cysts (meta-cercariae) caused by a trematode parasite in populations of Pacific treefrogs (*Hyla regilla*) and the Santa Cruz long-toed salamander (*Ambystoma macrodactylum croceum*). Experimental implantation of metacercariae-sized resin beads into the developing limb buds of the African clawed frog (*X. laevis*) resulted in limb malformations similar to those observed in the wild (Sessions and Ruth 1990).

Recently, Johnson *et al.* (1999) observed a correlation between the trematode *Ribeiroia ondatrae* and limb abnormalities in a number of frog species at several sites in northern California. To investigate further, the authors exposed larval Pacific treefrogs and western toads (*Bufo boreas*) to realistic numbers of cercariae (larvae) of the trematode *Ribeiroia* in the laboratory (Johnson *et al.* 1999, 2001a). This resulted in high frequencies (40–100%) of severe limb abnormalities, identical to those observed at field sites, including extra limbs, skin webbings, bony triangles, and missing or partially missing hind limbs (Figures 1 and 2). Similar experiments have yielded comparable results in northern leopard (*R. pipiens*) and wood frogs (*Rana sylvatica*) (Stopper *et al.* 2002). However, different species exhibit varying degrees of sensitivity to trematode infection, and there is also variation in how the deformities develop. For example, while extra limbs are the most common irregularity observed among infected Pacific treefrogs, severe skin webbings predominate in western toads (Johnson *et al.* 2001a).

Field experiments in Pennsylvania by Kiesecker (2002) corroborate results from the laboratory. Larval wood frogs were held in field enclosures with two different sizes of mesh. Amphibians in enclosures with the smaller mesh, which excluded cercariae of the trematode *Ribeiroia*, developed normally. However, in exclosures with mesh large enough to allowed cercariae to enter, severe limb abnormalities were observed.

Broad scale field surveys have further strengthened the connection between amphibian limb defects and *Ribeiroia* infection. Johnson *et al.* (2002) reported a significant association between *Ribeiroia* infection and frequency of malformations above baseline (>5%) in six amphibian species

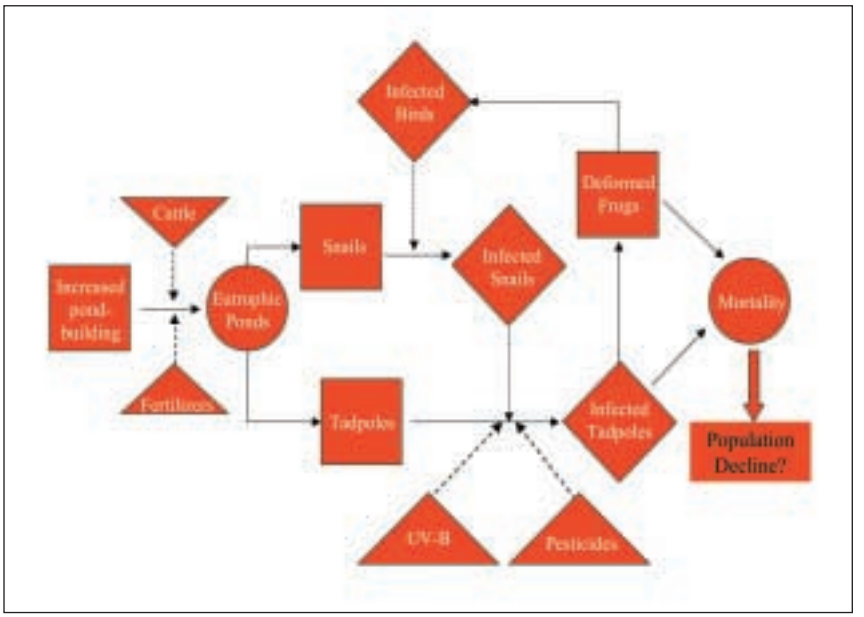

Figure 4. *Flow diagram illustrating the influence of parasites, artificial pond eutrophication, UV radiation, and pesticides on amphibian population declines and deformities.*

in five western US states. The average level of infection in a population was positively correlated with the frequency of developmental defects, which exceeded 90% at some field sites. More recently, *Ribeiroia* infection has also been implicated as a cause of deformity "hotspots" in the eastern and Midwestern US, including several of the Minnesota sites that first attracted the attention of the media and scientists alike (Sutherland in press; Lannoo *et al.* in press). Taken together, the parasite data make a convincing case that *Ribeiroia* infection is an important and widespread cause of amphibian abnormalities.

Even with good field observations and a growing data set based on experimental evidence, parasitic infection cannot be the cause of all amphibian limb malformations. In some places, deformed frogs are found where *Ribeiroia* is apparently absent. In addition, certain changes, including misshapen eyes, twisted internal organs, and some cases of missing limbs, are not caused by parasites – nor is parasitic infection likely to explain a high incidence of missing or partially missing legs in amphibians. Ultraviolet radiation can cause skin lesions, distortions of the eyes and body, and limb malformations. Some chemical contaminants also cause a very low frequency of hind limb irregularities. Predators, such as fishes, turtles, and invertebrates may bite the legs off tadpoles and adult amphibians. In some places, therefore, the introduction of non-native predators may be responsible for an increased incidence of frogs with missing legs (Johnson *et al.* 2001a).

■ Complexity

Even as we gain a more thorough understanding of the causes of amphibian deformities, we still need to address the most fundamental question; Why are they occurring with increasing frequency? No matter what the cause, the process almost certainly involves a complex interaction between several stressors. Many abnormalities can be explained by parasitic infection, but habitat alteration, chemical contaminants, and UV-B radiation may be at least indirectly involved, as part of a dynamic process that enables infection to occur more easily (Figure 4). Stressors are well known to affect rates of parasitic infection and disease (Lafferty and Kuris 1999).

In human and wildlife populations, diseases become more prevalent as changes occur in the ecology of the host–parasite relationship. For example, reforestation in the northeastern US has led to an increase in white-tailed deer populations and the consequent emergence of tick-borne Lyme disease. Damming African rivers has facilitated the spread of the trematodes that causes schistosomiasis. Over the past several decades we have witnessed the emergence and spread of other diseases such as hantavirus, Ebola, West Nile virus, dengue hemorrhagic fever, and AIDS, often due to human-mediated changes in the environment.

Certain diseases also have major impacts on amphibian populations. For example, outbreaks of some viruses, as well as the pathogenic oomycete *Saprolegnia* and the fungus that causes the fatal frog disease chytridiomycosis, seem to be increasing in frequency (Daszak *et al.* 1999; Blaustein and Kiesecker 2002). Similarly, *Ribeiroia* may be spreading as a consequence of changes in the ecology of its hosts. Johnson *et al.* (2002) reported that several regions in which numerous deformed amphibians and *Ribeiroia* were associated were in highly productive, artificial habitats, such as farm ponds that are used to water crops and cattle (Figure 5). Such habitats may have played an important role in the suspected proliferation of *Ribeiroia* and greater occurrences of amphibian deformities for several reasons. These systems are productive because of heavy fertilizer use and the presence of large quantities of cattle manure. This leads to increased algal growth and denser snail host populations that feed on algae (Figure 5). The number of artificial impoundments has risen dramatically since the 1940s, even as natural wetlands have been destroyed. Finally, the other necessary *Ribeiroia* hosts – birds and amphibians – are frequently found in such systems.

It is likely that additional stressors compromise amphibian immune systems, raising the chances of infection by pathogens such as *Ribeiroia*. Kiesecker (2002) reported a synergistic interaction between *Ribeiroia* infection and pesticide exposure. Amphibian larvae exposed to both *Ribeiroia* cercariae and low levels of pesticides showed

Courtesy of Steven Holt

Figure 5. *A severely eutrophic pond in western Montana, which over half of the resident Pacific treefrog population was found to have malformations. Inset: the planorbid snail hosts of Ribeiroia thrive under highly productive conditions.*

increased infection, decreased immune response, and a greater frequency of defects compared to amphibians exposed only to *Ribeiroia*. UV-B radiation, a known immunosuppressor (Tevini 1993), may also weaken amphibian defense mechanisms against disease, making *Ribeiroia* infection more likely to occur.

Ecological implications

The disappearance of amphibians, along with many other organisms, is part of a global biodiversity crisis. Massive mortality of amphibian eggs, larvae, and adults has been reported in some areas, and deformed amphibians only rarely survive to adulthood (Johnson *et al.* 2001b). Occasionally, amphibian abnormalities are associated with massive die-offs and declining populations (Hoppe in press). Malformations of the limbs impair mobility, decrease food intake, increase susceptibility to predators and parasites, and may eventually impact entire populations. Malformed frogs may actually benefit *Ribeiroia* by increasing the odds a frog will be consumed by a bird; the parasite cannot complete its life cycle until it arrives in an avian esophagus (Figure 3). As documented with other multi-host parasites, behavioral modification of the host may also increase *Ribeiroia's* fitness (Kuris 1997).

Whatever the cause of death, it is clear that deformed frogs do not survive to sexual maturity, and a high frequency of amphibian larvae may die as a direct result of *Ribeiroia* infection, even before they develop defects (Johnson *et al.* 1999). This may result in less viable populations, and severe population declines have been observed in at least one Minnesota site infested with *Ribeiroia* (Hoppe in press; Sutherland in press). However, there are currently few long-term data sets that directly link deformed amphibian numbers with population declines.

Solutions

Even though trematode parasitism is a likely explanation for many amphibian abnormalities, other factors are obviously involved. In this complex emerging problem, chemical contaminants, such as pesticides, or global environmental changes, such as increasing UV radiation, may compromise the immune system, leaving the animals vulnerable to infection (Figure 4). What is certain is that amphibians are subjected to multiple agents that stress them in a variety of ways, affecting both individuals and perhaps whole populations.

A major obstacle to understanding this issue is that some factors ignore political boundaries – for example, increasing levels of UV-B radiation and the spread of contaminants. This causes considerable problems for the implementation and enforcement of mitigation laws (Starke 2001). International treaties are necessary to address these problems. There has been some success in controlling anthropogenic ozone-destroying substances, thanks to the Montreal Protocol, the first multi-national effort to solve a worldwide environmental problem. In contrast, treaties to limit the production of gases that contribute to global warming, and those limiting the use of toxic substances, have been less successful. The Rotterdam Convention on the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides in International Trade is not yet in force (Starke 2001), and the Kyoto Protocol, which requires that industrial countries reduce emissions of carbon dioxide to help reduce global warming, is not currently supported by the US. On May 23, 2001, 127 nations adopted the Stockholm Convention on Persistent Organic Pollutants. Unfortunately, there are still hundreds of toxic chemicals polluting amphibian habitats.

Controlling the agents that lead to amphibian deformities may be easier on a local scale. It is important to quantify the proportion and types of abnormalities within a population. Long-term monitoring to assess population viability and key life history characteristics is necessary to address whether the malformations are contributing to population declines. If they are, it is critical to identify the contributing agents. We suggest abandoning the single-

factor approach, and instead designing field experiments that can detect interactions between several key factors.

Both long-term preventive solutions and more immediate, short-term efforts are important. For example, if trematode parasitism was identified as the main cause of amphibian deformations, solutions should concentrate on interrupting transmission between the snails and their amphibian hosts. Long-term solutions would include reducing nutrient inputs, more efficient use of fertilizers, and the reduction of cattle access to aquatic systems. Shoreline vegetation should be used as a riparian buffer, further lowering nutrient inputs, shading out excess algal growth, and reducing UV penetration. More efficient application of pesticides involves reducing applications when amphibians are most sensitive, such as during breeding events and early larval development. Controlling snail populations with biological control agents may also be an option.

The challenge is to unravel how the agents causing amphibian deformities interact with one another. This is a unique opportunity to deal with a complex emerging problem from a number of different angles. A coordinated and diverse array of scientists from many disciplines, including atmospheric scientists, ecologists, parasitologists, toxicologists, and developmental biologists, will be necessary to solve the problem.

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