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Amphibian declines: an immunological perspective

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Abstract

Many, but not all, amphibian populations have been declining on all six continents on which they live. Although habitat destruction, direct application of toxicants, and introduction of predators/competitors are obvious causes of amphibian declines, many amphibians are dying of infectious diseases in relatively pristine habitats on several continents. In this paper, we review the patterns of these disease outbreaks and the characteristics of amphibian immune systems. Hypotheses are presented to explain the apparent susceptibility of amphibians to these pathogens. Natural and man-made factors that can alter amphibian immune responses to pathogens are discussed. Additional research is needed on the biology of the specific pathogens, the pattern of immune responses they elicit, and the nature of environmental stressors that may increase susceptibility to infectious disease.

1. Introduction

Population sizes of many, but not all, amphibian species have experienced decline on six continents [1,2]. In some cases, extinctions of populations have occurred [3–17]. Although localized die-offs of amphibians were recorded before 1970 [18,19], major declines were documented in temperate areas beginning in the 1970's [3–12] and mass mortalities of amphibians in the tropics have been described more recently [13–17]. The global scope of these declines and the rapidity with which they have occurred have led to the suggestion that anthropogenic environmental disruption is at fault [1]. Habitat destruction, introduction of predators, and direct exposure to toxicants have certainly caused some amphibian declines [1]. The causes of mortality in such instances are fairly obvious. For instance, clear-cutting of forests or drainage of wetlands modify the thermal and hydric conditions beyond tolerance limits of amphibians, their young, and/or their insect prey. Most perplexing, however, are

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Nomenclature

ACTH	adrenocorticotropic hormone	TH	thyroid hormones
CH	corticosteroid hormones	TSH	thyroid stimulating hormone
CRH	corticotropin releasing hormone	UV-B	ultraviolet-B light
NK	natural killer		

instances in which environmental disruption is not clearly linked to population declines, particularly in relatively undisturbed areas such as national parks and wilderness areas of the western United States [4,6,7,9–11], and rain forests of Central America and Australia [13–16]. In an important number of these cases, infectious disease now appears to be the proximal cause of death. In these instances, no evidence yet exists that humans or anthropogenic environmental disruption have played any role in the evolution of these pathogens, movement of these pathogens from place to place, or susceptibility of amphibians to these pathogens. Infectious disease has influenced the population biology of animals and plants since early in their evolution [20]. However, recent outbreaks of disease in amphibian and in other animal taxa [21–25] should be given serious attention because they may signal environmental change on such a global scale that many groups of organisms, including humans, could be threatened. This paper will summarize the apparent patterns in pathogen-related amphibians declines and will review current hypotheses concerning the apparent failure of amphibian immune systems to resist disease agents.

2. Involvement of disease in recent amphibian declines

2.1. Pattern I: mass mortalities attributed to fungal infections

A number of mass mortalities of amphibians share many or all of the following characteristics: die-offs occur over geographically widespread areas; populations experience 50–100% mortality; declines are more pronounced at relatively higher altitudes (above 1500 and 400 m in temperate and tropical mountains, respectively) or relatively cool regions; only some of the total number of amphibian species in a locality experience population declines; mortality occurs principally among metamorphosed individuals, and infectious disease appears to be the direct cause of death [6,9,14–17].

This pattern is currently thought to be caused by fungal pathogens. For example, a number of stream-dwelling amphibians in tropical rainforests of eastern Australia and Central America have experienced recent population declines and/or extinctions [13–17]. Dead and dying frogs exhibited patches of abnormal epidermal sloughing [17]. Microscopic examination of the epidermis in affected areas revealed a non-hyphal, parasitic chytrid fungus (Chytridiomycota; Chytridiales). Healthy frogs exposed to unfiltered skin scrapings from diseased frogs became ill or died within 18 days, whereas unexposed control frogs remained healthy. Due to difficulties in culturing the fungus in this study, Koch's postulates were not fulfilled to establish this fungus as the causal agent. However, pathogenic bacteria, such as *Aeromonas hydrophila* and *Flavobacterium indologenes*, were isolated from less than 50% of the sick and dead frogs, and histological findings were not consistent with bacterial disease. No viruses, epidermal myxozoa, or protozoa were detected [17]. The association of this fungus with mortality in metamorphosed amphibians is explained by its tendency to attack keratinized structures, such as the epidermis of post-metamorphic individuals: some amphibian larvae have also been found with infected mouth parts, the only location in which they possess keratin. Museum specimens collected prior to the onset of amphibian declines in Central America and

Table 1
Apparent starting dates for mass mortalities of various species in North America. Studies in which the starting date can be verified because an observer was present in the field the year before and noted no mortalities are designated with ([a])

Location	Starting dates	Species	Reference
California	1974–1982	*Bufo canorus*	[10][a]
California	1979	*Rana muscosa*	[6]
Wyoming	early to mid-1970's	*Bufo baxteri*	[109]
Colorado	1974–1982	*Bufo boreas*	[9][a]
Colorado	1973	*Rana pipiens*	[4][a]
Manitoba	1975–1976	*Rana pipiens*	[8]
Alberta	1979	*Rana pipiens*	[3][a]

Queensland are currently being examined for the presence of chytrids. The results from this study will indicate when the organism was introduced to various geographic regions. It is unknown whether human activities and/or changes in environmental factors, such as global warming, have contributed to the spread of this pathogen from one locality to another.

A chytrid fungus has recently been isolated from superficial keratinocytes of two species of juvenile poison dart frogs (*Dendrobates* sp.) and one species of mature, adult tree frog (*Litorea caerulea*) held captive at the National Zoological Park in Washington, D.C. [26]. The fungus, named *Batrachochytrium dendrobatidis*, has been isolated, cultured, and used to transmit infection into healthy frogs. When these frogs became sick, the fungus was then reisolated from one of the infected frogs in order to fulfill Koch's postulates, verifying this fungus as the cause of death [27].

Fulfillment of Koch's postulates has established that extinctions of wild populations of Wyoming toads (*Bufo baxteri*) and disease outbreaks in captive individuals are due to primary infection by another fungus, *Basidiobolus ranarum* [28]. Identification of this fungus is currrently being reevaluated to determine if it is related to, or the same as, the chytrid fungus responsible for other amphibian deaths. Mortality of toads was associated with mycotic dermatitis, characterized by reddened and thickened skin with abnormal sloughing. Secondary infection with pathogenic bacteria, particularly *Aeromonas hydrophila*, was common in many of the infected toads [28].

The characteristics outlined above as Pattern I were exhibited in a number of geographically-widespread amphibian population declines that began in the 1970's in western United States and Canada (Table 1). Because the technology and expertise necessary to diagnose viral and fungal pathogens in amphibians are relatively recent, sick amphibians in these die-offs were not surveyed for all potential pathogens. As a result, pathogenic bacteria or undefined "diseases" were thought to cause some of these early population declines [6,9,10]. Chytrid fungi have now been identified in 2 of 12 museum specimens of *Bufo canorus* collected in the California Sierra Nevada during the period of mass mortality in the 1970's (D. Green and C. Kagarise Sherman, pers. comm.) and in *Rana pipiens* (D. Green, C. Carey and S. Corn, unpubl. data) collected in the Colorado Rockies in a mass mortality event in the 1970's [4,10]. While Koch's postulates cannot be verified, of course, using preserved fungi from museum specimens, the degree of infection of the preserved specimens is thought to be sufficient to have been lethal (D. Green, pers. comm.). Regrettably, specimens of a few of the other species in Table 1 cannot be evaluated for the presence of chytrids since none were preserved during their mass die-offs. However, the relation between mass mortalities of *Bufo canorus, Rana pipiens*, and *Bufo baxteri* [28] in the 1970's and fungal infections and the similarities in patterns of die-offs among the groups in Table 1 leads to the speculation that fungal infections could have been the primary cause of death of most, if not all, those mass die-offs.

The possibility that a chytrid fungus was responsible for the widespread die-offs of toads and frogs in western North American during the 1970's raises many important questions. Since a few populations of some of the species decimated during the 1970's survived and have persisted in a few localities [3,7,8,11], did the fungus "miss" these populations or did these populations have some form of immunity against it? In most cases, other amphibian species that occupied the same

habitats in which toads and frogs died were unaffected (for instance, see [7,9]). Were the surviving species immune to the fungus, and if so, how do their immune mechanisms differ from those of the affected toads and frogs? Is the fungus responsible for die-offs in Central America and Australia genetically related to the one(s) that could have resulted in the western North American mass mortalities in the 1970's or are pathogenic fungi evolving de novo in various habitats?

Recent mass mortalities of infectious disease in *Rana* in southern Arizona have been just linked by histological evidence to a chytrid fungus (M. Sredl, pers. comm.). If this observation signals an evolution of a new pathogenic fungus or a resurgence of the pathogenic fungus from the 1970's, more amphibian mass mortalities might be expected in coming years. These findings underscore the need to learn more about the biology of this pathogen and its interaction with amphibians.

2.2. Pattern II: mass mortalities attributed to iridoviruses

A second pattern of disease outbreaks has been attributed to iridovirus infections: mortalities are usually restricted to small geographical areas, even a single pond; larval, metamorphosing, or post-metamorphic stages of amphibians may succumb; outbreaks occur frequently, but not exclusively, in areas disturbed by human impacts, and infections occur especially at high population densities. This pattern is typified by recent disease outbreaks in *Rana temporaria* in England [29] and *Ambystoma tigrinum stebbinsi* in Arizona [30]. A similar pattern has been observed in scattered mass mortalities of metamorphosing *Ambystoma tigrinum* at several locations in the Colorado Rockies (Carey et al., unpubl. data).

Two disease syndromes in *Rana temporaria* have been described: one in which dermal ulcerations were the predominant disease manifestation and the other was characterized by systemic hemorrhages. After an extensive study of both sick and dead frogs that included histology, culture for bacterial pathogens, and examination for viruses by electron microscopy the authors concluded that the most likely causes of these disease syndromes were either primary iridovirus infection only, or primary iridovirus infection followed by secondary infection with opportunistic bacteria (usually *Aeromonas hydrophila*) [29].

In the examination of salamander deaths in Arizona [30], disease could be transmitted in the laboratory from sick individuals to healthy ones by exposure to water in which the diseased animals had been housed. The water contained a filterable agent (passed through a 0.45 μm filter) that produced cytopathic effects on two fish cell lines. The infected cells containing virus were used to transmit the disease to uninfected salamander hosts. Virus was reisolated from the infected salamanders and was capable of introducing cytopathic effects in a new set of freshly cultured fish cells. Hemolytic bacteria were isolated from 71% of diseased animals but exposures of healthy animals to bacterial concentrations of up to 10^8 ml^{-1} did not cause disease.

Iridoviruses have been isolated from sick Canadian tiger salamanders collected during a recent mass-mortality event [31] and they have been recently detected in tissues of sick salamanders (D. Docherty, L. Creekmore, and L. Glaser, pers. comm.) in the same locality in which mass die-offs of *Ambystoma tigrinium* occurred in 1982–1986 [32]. A bacterial pathogen was thought to be responsible for the 1982–1986 die-offs [32]. Although it is tempting to conclude that the earlier die-off might have been caused by iridoviruses, examination of a small sample of preserved specimens revealed no viruses (D. Green, pers. comm.). Studies are underway to study the taxonomic relationships among the iridoviruses causing these amphibian deaths in various localities and to determine how the virus can be transmitted from locality to locality.

2.3. Disease outbreaks attributed to bacterial infections and other possible agents

Although current evidence from recent amphibian declines suggests that bacterial infections may be secondary to fungal or viral infections,

the possibility that bacterial infections alone have caused mass mortalities of amphibians must not be totally ignored. For instance, *Aeromonas hydrophila* have been implicated, through fulfilment of Koch's postulates, as the causative pathogen in a localized die-off of American toads, *Bufo americanus* [18]. Since facultatively pathogenic bacteria can be present on the skin, in the digestive tract, and in tissues usually considered sterile (liver, kidney) in frogs showing no pathological characteristics or other evidence of general contamination [33,34], bacterial infection could cause death particularly in situations associated with crowding, exposure to toxic agents, or hibernation [35,36]. Furthermore, recent evidence that *Aeromonas hydrophila* increase growth rate many-fold after exposure to vertebrate "stress" hormones, such as norepinephrine, indicates that facultatively pathogenic bacteria may be able to detect when their hosts are under "stress" and vary their virulence accordingly [37].

No current evidence implicates eukaryotic parasites as a primary cause of amphibian declines.

3. Amphibian immune mechanisms for disease resistance

Resistance of amphibians to pathogens involves the innate immune system as well as the adaptive immune system. Although the amphibian immune system has received a great deal of study, the defenses against fungal and viral attacks are not well characterized. In the following sections, we will review what is known about defenses against these pathogens, along with a general summary of the amphibian immune system.

3.1. Innate immunity

The innate immune system provides rapid, non-specific protection until the adaptive immune response can be mobilized. The first defense against invasions of pathogens through the skin and digestive tract is most likely anti-microbial peptides. These peptides are small (20–46 amino acid residues), basic, and amphipatic. They have activity towards a variety of microorganisms, including bacteria, yeast, and fungi [38,39]. The chemical structures, biosynthesis, mechanisms of action, and activities towards various microorganisms of amphibian anti-microbial peptides are just beginning to be characterized. Nothing is currently known about how and if environmental changes, such as temperature, pH, hydration of the skin, exposure to toxicants, etc., affect the ability of these peptides to protect amphibians from microbial pathogens.

Like all vertebrates, amphibians have phagocytic cells, primarily macrophages and neutrophils, that can directly phagocytize a pathogen [40–42]. Amphibians also share with other vertebrates a complement system that can kill bacteria directly by activation of the alternative pathway of complement and development of the membrane attack complex [43,44]. Complement also functions in conjunction with antibody. Thus, antibody-coated pathogens bind complement components via the classical pathway [45]. Pathogens can be killed directly by the membrane attack complex or by macrophages that are assisted in binding and phagocytosis by the antibody coating.

Natural cytotoxicity provided by natural killer (NK) cells is another element of the innate immune system of vertebrates. NK cells provide an immediate cytotoxic response against virus-infected or tumor targets in unprimed animals (those not previously immunized with the antigens in question). Splenocytes of adult South African clawed frogs (*Xenopus laevis*) display NK-like activity toward tumor cell targets from allogenic (different individuals of the same species) animals. Spleen cells from larvally thymectomized adult frogs exhibit this activity [46,47], but it appears to be lacking in tadpole spleens [47].

3.2. Adaptive immunity

The adaptive immune response requires time to be activated following the detection of a foreign antigen. It is highly specific for a given pathogen and results in the generation of mem-

ory cells that can respond swiftly to a repeated antigenic insult. Nothing is known about how or whether this system functions in regard to fungal pathogens.

Although we know a good deal about adaptive immunity in anuran and urodelean amphibians, most of that information has been obtained from experiments conducted with *Xenopus laevis* and the axolotl (*Ambsytoma mexicanum*). If the immune system of *Xenopus* adults is similar to that of other species of frogs and toads [48,49] then, in general, the anuran immune system has almost all the components of mammalian immune systems. Anuran immune systems have T- and B-lymphocytes [50] that express cell surface T-cell receptors (TCR) and immunoglobulin (Ig) receptors, respectively, Ig isotype heterogeneity, leukocyte-derived cytokines [51–53], and major histocompatibility complex (MHC) class I and class II genes [54]. MHC-restricted cytotoxic and helper T-cell responses have been characterized in adults [55–57]. Although *Xenopus* lacks the mammalian equivalent of lymph nodes and a lymphopoietic bone marrow, this species does have a thymus and spleen, important central and peripheral lymphoid organs, respectively.

While *Xenopus* has served as the prototypic frog for immunologic studies of anurans, the axolotl has served as the urodele model. Axolotls, like *Xenopus*, have T-cells with a TCR [58] and B cells that express surface Ig receptors and produce antibody [59]. The axolotl MHC with class I [60] and II-encoded molecules has been described [61] but nothing is known about its role in antigen presentation and recognition. Although salamanders have these and other basic hallmarks of adaptive immunity, the in vivo antibody response to a variety of antigens [62] as well as to alloantigens [63] is typically far less robust than that of anurans. No explanation exists for this apparent functional difference between the immune system of frogs and salamanders, nor is it known whether this difference causes salamanders to be more susceptible to pathogens in nature than anurans.

4. Hypotheses to explain apparent recent increases in susceptibility of amphibians to infectious disease

Given that amphibians have a rather sophisticated adaptive immune system, mass die-offs of anuran species due to disease cannot simply be attributed to the possibility that amphibians have a "poor" immune system. Some hypotheses (which may not be mutually exclusive) have been proposed to explain these phenomena. These hypotheses orginated from a variety of sources, including publications (cited with the specific hypothesis), formal presentations at scientific meetings, and informal conversations with a variety of scientists. It should be noted that data to support or reject these hypotheses are minimal at best.

1. Amphibians are exposed to new, highly virulent pathogens that kill them before their immune defenses can be adequately mobilized. These pathogens might have recently evolved from non-pathogenic forms in various habitats. Alternatively, human activities such as fishing, fish stocking, etc. or wind/weather patterns may be introducing pathogens into habitats in which they were not formerly found.
2. A variant of hypothesis 1 is that the "new" pathogens are immunosuppressive, such as an immunosuppressive virus like HIV, or produce immunosuppressive compounds, such as a fungal metabolite like cyclosporin.
3. Alteration in environmental conditions (i.e. temperature, moisture, etc.) or contaminant concentrations might alter the proportions of various soil and water microorganisms in a way that a previously rare pathogen in a given habitat becomes prevalent.
4. Environmental changes might "stress" the host, with the result that the production of host stress hormones cause an increase in the virulence (i.e. growth rate) of the pathogens [37].
5. Exposure to environmental contaminants directly diminishes components of the innate and/or adaptive immune system (e.g., leukocyte toxicity, skin defenses). Immunosuppression,

in turn, results in increased vulnerability to disease and/or opportunity for pathogens to switch to new hosts [9].
6. Single or multiple combinations of sublethal environmental changes cause neuroendocrine changes reflecting ''stress'', which in turn cause immunosuppression [9].

5. Factors that could modulate amphibian resistance to pathogens

Although a growing scientific literature exists on iridoviruses in fish diseases, very little is known about the fungal and viral pathogens causing widespread amphibian mortalities. Areas that need further study are: how these pathogens are spread from place to place, how they penetrate the skin, intestinal tract, etc. of amphibians, whether these pathogens can invade and replicate faster than the immune system can mount an effective defense, and how other natural and/or anthropogenic factors influence the interaction between pathogens and the immune system of amphibians? We now review what is known about natural and potential man-made environmental changes that could modulate amphibian immune function.

5.1. Natural modulators

Natural immunomodulating factors, such as developmental changes in immune competence and variations in body temperature, have been present throughout the evolution of amphibians. While these are not expected to directly cause recent amphibian declines due to infectious disease, they may act synergistically with other factors to increase susceptibility to disease.

5.1.1. Developmental changes in amphibian immune function

Various components of the amphibian immune system mature at different stages of development. Full immunocompetence is not achieved, at least for the few species studied, until after metamorphosis [48,49,64,65]. Information about the life stage at which defense mechanisms against various pathogens mature, coupled with knowledge of the stages at which various pathogens strike amphibians, will together provide important clues about the mechanism by which the pathogens successfully evade the immune defenses of the animal. Some, but not all, of the amphibian die-offs are specific to certain life stages (see Patterns I and II above). While fungi preferentially target metamorphosed individuals [17,28], iridoviruses successfully attack larvae, metamorphosing and post-metamorphic individuals [29,30].

Unfortunately, little is known about the timing and process of the development of amphibian immune systems except in *Xenopus* [48]. Very little is known about metamorphosis-induced changes in immunocompetence in urodeles, largely because the model organism, *Ambystoma mexicanum*, is neotenic. Although *Xenopus* larvae are immunocompetent, they are naturally MHC class I deficient. As a result, their immune system may have difficulty recognizing a viral pathogen. Surface expression of class I molecules doesn't appear until metamorphosis when there is a profound remodeling of the immune system [64–66]. More precisely, MHC class I antigens are first detected on erythrocytes and on splenic leukocyte populations at the prometamorphic and climax stages of metamorphosis, respectively [67,68]; class I expression on *Xenopus* thymocytes is not detectable until metamorphic climax [68,69]. Thus, although the immune system of larval *Xenopus* displays many features of the adult, such as specific antibody responses with immunoglobulin isotype switching [70,71] and rejection of MHC disparate adult skin grafts [72], their immune system operates with very low levels of cell surface expression of MHC class I antigens. The extent to which characteristics of larval *Xenopus* typify those of larval anurans in general is unknown. It is also unknown for *Xenopus* and other species whether in the absence of class I-restricted T-cells, class II molecules could serve in larval cytotoxic immune reactions. It is known, however, that during *Xenopus* larval life, MHC class II antigen expression in the periphery is predominantly expressed on B-lymphocytes

and accessory cells [73,74], as in adult mice and rats. However, after metamorphosis, class II antigens are expressed constitutively on virtually all mature peripheral T- as well as B-cells [73,74]. Therefore, while the immune systems of the larval forms are competent to defend against potential pathogens during development in an aquatic environment, they are not equivalent to the mature immune systems that develop after metamorphosis.

When metamorphosis is inhibited by treating tadpoles with sodium perchlorate to block normal thyroid function, the adult-type MHC class II-positive peripheral T-cell population does not develop. Thus, maturation of this population appears to depend on normal metamorphosis [74]. We now know that the increased concentrations of metamorphic hormones, principally thyroid hormones (TH) and corticosteroid hormones (CH), orchestrate the loss or reorganization of many tissues and organ systems, including those of the immune system [64,65]. Indeed, immune system reorganization may serve to eliminate unnecessary lymphocytes that could be destructive if they recognized newly emerging adult-specific antigens on the adult tissues. Increased corticosteroids during metamorphosis appear to induce apoptosis of susceptible lymphocytes [75–77]. This cell death can be inhibited in vitro or in vivo by the corticosteroid receptor antagonist, RU486 [76,77]. A coordinated increase in both TH and CH at metamorphosis may be common to all amphibians that undergo metamorphosis. Current evidence suggests that the central hypothalamic mediator that induces pituitary production of both thyroid stimulating hormone (TSH) and adrenocorticotropic hormone (ACTH) in larval amphibians is corticotropin-releasing hormone (CRH) [78–80].

The conditions under which tadpoles undergo metamorphosis may have a profound effect on their subsequent immunocompetence. Previous studies on the effect of various feeding regimens on the time to metamorphosis and weight at metamorphosis suggests that when tadpoles are provided with less than an optimal amount of food, they metamorphose later and at a smaller size [81–84]. In laboratory studies, we observed that *Xenopus* tadpoles forced to metamorphose precociously had more dramatic lymphocyte losses than controls metamorphosing on a normal schedule [85]. These findings suggest that when animals undergo metamorphosis at a smaller than ideal size, the immune system may be seriously compromised. Thus, habitat alteration leading to development and metamorphosis in less than optimal conditions could cause greater than normal losses of lymphocytes and a concomitant increase in susceptibility to disease during and after this critical transitional time [65].

5.1.2. Ambient temperature

Cellular and humoral immunity of ectotherms are strongly influenced by ambient temperature [86]. Prolonged cold, such as experienced during hibernation in temperate amphibians, leads to substantial diminution of some immune capacities [45,87–90]. The degree to which such cold-induced immunosuppression puts amphibians at risk depends upon the temperature-dependence of the virulence of pathogens. The relationship between immunocompetence of various amphibians and the virulence of pathogens needs additional study, both during exposure to prolonged cold and sudden temperature changes. Although fluctuations in ambient temperature have been a part of the physical environment of amphibians throughout their evolution, the interrelations among climate changes, amphibian immune systems, and pathogens need to be evaluated. For instance, the altitudinal increase in the zero degree isotherm in Central American tropical mountains over the last three decades [91] may affect virulence of particular pathogens to a different degree than the immune defenses of amphibians.

5.1.3. Man-made modulators

The term ''stress'' or ''stressor'' is often used in conjunction with environmental changes. ''Stress'' has a number of connotations and has probably been so overused as to obscure its meaning. Some use the term as it was originally defined by Hans Selye to mean that imposition of a ''stressor'' causes a deviation from homeo-

stasis that the animal attempts to adapt to with complex neurological and neurochemical changes in an attempt to restore homeostatic equilibrium [92,93]. By this definition, an environmental change, such as variation in pH or UV light, would not qualify as a stressor unless it results in demonstrable neuroendocrine changes within the animal. Ecologists, however, do not limit their use of "stress" to the effect of stressors that elicit secretion of neuroendocrine hormones. Rather they use the term "stress" in a broad sense [94] to mean any kind of deleterious or injurious environmental change to which a species has not evolved the capacity to compensate fully. Environmental changes, "stressful" or not by any definition, can directly impact immune function through action on the immune system itself or indirectly through hormonal secretions associated with neuroendrocrine responses to "stress" [92,93]. As a result, it is important to know how man-made environmental changes affect immune function in amphibians.

5.1.4. Psychosocial stressors

Outbreaks of infectious disease in *Ambystoma tigrinum* in Arizona occurred most frequently at high density levels [30]. Such high levels may be a natural, periodic consequence of fluctuations in population size in many amphibian species [95]. However, as habitat destruction of many amphibians limits the availability of traditional breeding sites, migration pathways, and hibernacula, population densities may rise at inappropriate times in the annual cycle. Although laboratory studies show that housing conditions can modulate immunity of mammals [96], the relationships among crowding (or any other psychosocial stressor), immune system competence, and contagion is unclear for amphibians.

5.1.5. Ultraviolet light

Most amphibian larvae are exposed to some degree of natural sunlight, depending on the amount of vegetation and dissolved organic content of the ponds in which they are developing. As amphibians metamorphose, many species become nocturnal or active diurnally in shade. Larvae of a few species, especially those living at high altitude where UV-B levels in air are higher than at lower altitudes, seek shallow, warm water during the day (Little et al., pers. comm.) and metamorphosed individuals are diurnal and bask in the sun [97]. As the reduction in the ozone layer results in more ultraviolet-B (UV-B) reaching the ground, particularly at high altitudes, UV-B exposures may exceed levels for which amphibian protective mechanisms have evolved. Exposure to sublethal levels of UV-B can suppress immune function in mammals, including humans (reviewed in [98]). UV-B damage to immune systems is expressed both immediately and over a period of years (i.e., skin cancers and cataracts [98]). Even exposure of mice to relatively low levels of UV-B impairs their ability to reject tumors (leading to the development of cutaneous carcinomas) and their ability to detect contact antigens (skin cells lose the ability to detect and fight foreign pathogens), with the result that fungal, bacterial, viral, and parasitic skin infections can easily occur after UV-B damage [99–102]. It is currently unknown whether these laboratory observations on nocturnal mice can be directly extrapolated to amphibian species, particularly those that have evolved under exposure to solar radiation, and how sublethal exposure to UV-B affects immune systems of larvae and post-metamorphic individuals.

5.1.6. Xenobiotics

Exposures to heavy metals and various man-made chemicals compromise immune function in mammals [103] and other vertebrate species [104]. Relatively few studies of the effect of such putative toxicants on immunity in "lower" vertebrates (mostly fish) exist [104–107]. A large body of literature exists on toxic effects of various xenobiotics on 96-h amphibian embryos [108]. However, since metamorphosed amphibians and older larvae are those principally dying of infectious disease in the field, more study is necessary to determine the potential impact of these environmental factors in causing diseases in amphibians.

6. Concluding remarks and recommendations for future research

As discussed extensively in this review, there is now compelling evidence for mass deaths among amphibian populations in diverse geographic locations due to disease outbreaks. The patterns of losses appear to exceed normal population fluctuations and suggest one or more globally emerging disease epidemics may place existing amphibian populations at risk of extinction. Although amphibians have effective and diverse immune defense mechanisms, the failure of these defenses to prevent infections suggests that new disease agents may have recently emerged or that environmental stressors may be compromising the response of amphibian immune systems. Fundamental research into the nature of specific causative pathogens, the pattern of immune responses they elicit, and the nature of environmental stressors that may contribute to disease susceptibility is needed. Research into the immune defenses against a specific pathogen should include studies of innate as well as adaptive immune defense mechanisms and should identify susceptible life stages. To identify exogenous stressors that may contribute to suppression of immunity and increased susceptibility to disease, model amphibians should be selected for initial laboratory studies to determine sensitive immunological parameters. Such studies should be coupled with field collections and field monitoring for presence of pathogens and possible stressors. Understanding the effects of stressors on host resistance to each selected pathogen following controlled laboratory or field exposures is necessary to demonstrate the impact of each suspected stressor, acting alone or in concert, on survival of selected populations.

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References

[1] Phillips K. Where have all the frogs and toads gone? BioScience 1990;40:422–4.
[2] Vial JL, Saylor L. The Status of Amphibian Populations. IUCN/SSC Declining Amphibians Populations Task Force, Working Document No. 1 IUCN 1993.
[3] Roberts WE. What happened to the leopard frogs? Alberta Nat 1981;11:1–4.
[4] Corn PS, Fogleman JC. Extinction of montane populations of the northern leopard frog (*Rana pipiens*) in Colorado. J Herpetol 1984;18:147–52.
[5] Heyer WR, Rand AS, da Cruz Goncalves CA, Peixoto OL. Decimations, extinctions, and colonizations of frog populations in southeast Brazil and their evolutionary implications. Biotropica 1988;20:230–5.
[6] Bradford DF. Mass mortality and extinction in a high-elevation population of *Rana muscosa*. J Herpetol 1991;25:174–7.
[7] Bradford DF, Graber DM, Tabatabai F. Population declines of the native frog, *Rana muscosa*, in Sequoia and Kings Canyon National Parks, California. Southwestern Nat 1994;39:323–7.
[8] Koonz W. Amphibians in Manitoba. In: Bishop CA, Pettit KE, editors. Declines in Canadian Amphibian Populations: Designing a National Monitoring Strategy. Ottawa, Ontario: Canadian Wildlife Service, 1992. p. 19–20.
[9] Carey C. Hypothesis concerning the causes of the disappearance of boreal toads from the mountains of Colorado. Cons Biol 1993;7:355–62.

[10] Kagarise Sherman C, Morton ML. Population declines of Yosemite toads in the eastern Sierra Nevada of California. J Herpetol 1993;27:186–98.
[11] Drost CA, Fellers GM. Collapse of regional frog fauna in the Yosemite area of the California Sierra Nevada. Cons Biol 1996;10:414–25.
[12] Beebee TJC, Flower RJ, Stevenson AC, Patrick ST, Appleby PG, Fletcher C, Marsh C, Natkanski J, Rippey B, Battarbee RW. Decline of the natterjack toad, *Bufo calamita*, in Britain: paleoecological, documentary and experimental evidence for breeding site acidification. Biol Cons 1990;53:1–20.
[13] Pounds JA, Crump ML. Amphibian declines and climate disturbance: the case of the golden toad and the harlequin frog. Cons Biol 1994;8:72–85.
[14] Laurance WF, McDonald KR, Speare R. Epidemic disease and the catastrophic decline of Australian rain forest frogs. Cons Biol 1996;10:406–13.
[15] Lips KR. Decline of a tropical montane amphibian fauna. Cons Biol 1998;12:106–17.
[16] Lips KR. Mass mortality and population declines of anurans at an upland site in western Panama. Cons Biol 1999;13:117–25.
[17] Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, Slocombe R, Ragan MA, Hyatt AD, McDonald KR, Hines HB, Lips KR, Marantelli G, Parkes H. Chytridiomycosis causes amphibian mortality associated with population declines in the rainforests of Australia and Central America. Proc Natl Acad Sci USA 1998;95:9031–6.
[18] Dusi JL. The natural occurrence of "redleg", *Pseudomonas hydrophila*, in a natural population of American toads, *Bufo americanus*. Ohio J Sci 1949;49:70–1.
[19] Hunsaker D, Potter FE. "Red leg" in a natural population of amphibians. Herpetologica 1960;16:285–6.
[20] Anderson RM, May RM, editors. Population Biology of Infectious Diseases. Berlin: Springer-Verlag, 1982.
[21] Lahvis G, Wells RS, Casper D, Via CS. In vitro lymphocyte response of bottlenose dolphins (*Tursiops truncatus*). Mitogen-induced proliferation. Marine Environ Res 1993;35:115–9.
[22] Osterhaus AD, Vedder EJ. Identification of virus causing recent seal deaths. Nature 1988;335:20.
[23] Schumacher IM, Brown MB, Jacobson ER, Collins BR, Klein PA. Detection of antibodies to a pathogenic mycoplasms in desert tortoises (*Gopherus assizii*) with upper respiratory tract disease. J Clin Microbiology 1993;30:1454–6.
[24] Herbst LH, Klein PA. Green turtle fibropapillomatosis: challenges to assessing the role of environmental cofactors. Environ Health Perspect 1995;103:27–30.
[25] Littler MM, Littler DS. Impact of CLOD pathogen on Pacific coral reefs. Science 1995;267:1356–60.
[26] Pessier AP, Nichols DK, Longcore JE, Fuller MS. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* ssp.) and White's tree frogs (*Litorea caerulea*), J Vet Diag Invest 1999;11:194–9.
[27] Longcore JE, Pessier AP, Nichols DK. *Batrachochytrium dendrobatidis* gen. and sp. Nov., a chytrid pathogenic to amphibians. Mycologia 1999;91:219–27.
[28] Taylor SK, Williams ES, Thorne ET, Mills KW, Withers DI. Causes of mortality of the Wyoming toad (*Bufo baxteri*). J Wildlife Diseases 1999;35:49–57.
[29] Cunningham AA, Langton TES, Bennett PM, Lewin JF, Drury SEN, Gough RE, MacGregor SK. Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). Phil Trans R Soc Lond B 1996;351:1539–57.
[30] Jancovich JK, Davidson EW, Morado JF, Jacobs BL, Collins JP. Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi*. Dis Aquat Org 1997;31:161–7.
[31] Bollinger TK, Mao J, Schock D, Brigham RM, Chinchar VG. Iridovirus of tiger salamanders in Saskatchewan: pathology, isolation and molecular characterization of a novel ranavirus. J Wildlife Diseases 1999; (in press).
[32] Worthylake KM, Hovingh P. Mass mortality of salamanders (*Ambystoma tigrinum*) by bacteria (*Acinetobacter*) in an oligotrophic seepage mountain lake. Great Basin Naturalist 1989;49:364–72.
[33] Hird DW, Diesch SL, McKinnell RG, Gorham E, Martin FB, Kurtz SW, Dubrovolny C. *Aeromonas hydrophila* in wild-caught frogs and tadpoles (*Rana pipiens*) in Minnesota. Lab Anim Sci 1981;31:166–9.
[34] Meteyer CU. 1998 Diagnostic findings associated with malformed frogs in Minnesota: Summer 1997. Preliminary Report. USGS Project Report, NWHC-BRD-USGS.
[35] Carr AH, Amborski RL, Culley DD, Amborski GF. Aerobic bacteria in the intestinal tracts of bullfrogs (*Rana catesbeiana*) maintained at low temperatures. Herpetologica 1976;32:239–44.
[36] Taylor S, Green DE, Wright KM, Whitaker BR. Bacterial diseases. In: Wright K, Whitaker B, editors. Amphibian Medicine and Captive Husbandry. Malabar, FL: Kreiger Publ, 1999 (in press).
[37] Kinney KS, Austin CE, Morton DS, Sonnenfeld G. Catecholamine enhancement of *Aeromonas hydrophila* growth, Micro Path 1999; (in press).
[38] Jacob L, Zasloff M. Potential therapeutic applications of magainins and other antimicrobial agents of animal origin. In: Marsh J, Goode JA, editors. Antimicrobial Peptides, vol. 186, 1994. p. 197–223 Ciba Foundation Symposium 1994.
[39] Nicolas P, Mor A. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Ann Rev Microbiol 1995;49:277–304.
[40] Manning MJ, Horton JD. RES structure and function of the amphibia. In: Cohen N, Sigel MM, editors. The Reticuloendothelial System, a Comprehensive Rreatise,

vol. 3, Phylogeny and Ontogeny. New York: Plenum, 1982. p. 423–59.
[41] Hadji-Azimi I, Coosemans V, Canicatti C. Atlas of adult *Xenopus laevis laevis* hematology. Dev Comp Immunol 1987;11:807–74.
[42] Plytycz P, Potter S, Cohen N, Bayne C. In vitro fusion of newt peritoneal macrophages. J Exp Zool 1988;20:319–23.
[43] Jensen LB, Koch C. An assay for complement factor B in species at different levels of evolution. Dev Comp Immunol 1991;15:173–9.
[44] Kato Y, Salter-Cid L, Flajnik MF, Kasahara M, Namikawa C, Sasaki M, Nonaka M. Isolation of the *Xenopus* complement factor B complementary DNA and linkage of the gene to the frog MHC. J Immunol 1994;153:4546–54.
[45] Green N, Cohen N. Effect of temperature on serum complement levels in the leopard frog *Rana pipiens*. Dev Comp Immunol 1977;1:59–64.
[46] Horton TL, Ritchie P, Watson M, Horton JD. NK-like activity against allogeneic tumor cells demonstrated in the spleen of control and thymectomized *Xenopus*. Immunol Cell Biol 1996;74:365–73.
[47] Horton TL, Ritchie P, Watson MD, Horton JC. Natural cytotoxicity towards allogenic tumor targets in *Xenopus* mediated by diverse splenocyte populations. Dev Comp Immunol 1998;22:217–30.
[48] Du Pasquier L, Schwager J, Flajnik MF. The immune system of *Xenopus*. Ann Rev Immunol 1989;7:251–75.
[49] Du Pasquier L. Evolution of the immune system. In: Paul WE, editor. Fundamental Immunology. New York: Raven Press, 1993. p. 199–233.
[50] Bleicher PA, Cohen N. Monoclonal anti-IgM can separate T-cell from B-cell proliferative respones in the frog *Xenopus laevis*. J Immunol 1981;127:1549–55.
[51] Watkins D, Cohen N. Mitogen-activated *Xenopus laevis* lymphocytes produce a T-cell growth factor. Immunology 1987;62:119–25.
[52] Watkins D, Parsons SC, Cohen N. A factor with interleukin-1-like activity is produced by peritoneal cells from the frog, *Xenopus laevis*. Immunology 1987;62:669–73.
[53] Haynes L, Cohen N. Further characterization of an interleukin-2-like cytokine produced by *Xenopus laevis* T lymphocytes. Dev Immunol 1993;23:1–23.
[54] Flajnik MF, Du Pasquier L. The major histocompatibility complex of frogs. Immunol Rev 1990;150:47–63.
[55] Blomberg B, Bernard CCA, Du Pasquier L. In vitro evidence for T–B lymphocyte collaboration in the clawed toad, *Xenopus*. Eur J Immunol 1980;10:869–76.
[56] Flajnik MF, Du Pasquier L, Cohen N. Immune responses of thymus/lymphocyte embryonic chimeras. Studies on tolerance and MHC restriction in *Xenopus*. Eur J Immunol 1985;15:540–7.
[57] Harding FA, Flajnik MF, Cohen N. MHC restriction of T cell proliferative responses in *Xenopus*. Dev Comp Immunol 1994;17:425–37.
[58] Fellah JS, Kerfourn F, Guillet F, Charlemagne J. Conserved structure of amphibian T-cell antigen receptor B chain. Proc Natl Acad Sci (USA) 1993;90:6811–4.
[59] Tournefier A, Fellah JS, Charlemagne J. Monoclonal antibodies to axolotl immunoglobulins specific for different heavy chains isotypes expressed by independent lymphocyte subpopulations. Immunol Letters 1988;18:145–8.
[60] Sammut B, Laurens V, Tournefier A. Isolation of MHC class I cDNAs from the axolotl *Ambystoma mexicanum*. Immunogenetics 1997;45:285–94.
[61] Kaufman J, Völk H. The salamander immune system: right and wrong. Axolotl Newsletter 1994;23:7–23.
[62] Ching Y, Wedgwood R. Immunologic responses in the axolotl, *Siredon mexicanum*. J Immunol 1967;99:191–200.
[63] Cohen N. Chronic skin graft rejection in the Urodela. I. A comparative study of the first- and second-set allograft reactions. J Exp Zool 1968;167:37–48.
[64] Rollins-Smith LA, Cohen N. Metamorphosis: an immunologically unique period in the life of the frog. In: Gilbert LI, Tata JR, Atkinson BG, editors. Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells. New York: Academic Press, 1996. p. 625–46.
[65] Rollins-Smith LA. Metamorphosis and the amphibian immune system. Immunol Rev 1998;166:221–30.
[66] Flajnik MF, Hsu E, Kaufman JF, Du Pasquier L. Changes in the immune system during metamorphosis of *Xenopus*. Immunol Today 1987;8:58–64.
[67] Flajnik MG, Du Pasquier L. MCH class I antigens as surface markers of adult erythrocytes during the metamorphosis of *Xenopus*. Devel Biol 1988;128:198–206.
[68] Rollins-Smith LA, Flajnik MF, Blair P, Davis AT, Green WF. Involvement of thyroid hormones in the expression of MHC class I antigens during ontogeny in *Xenopus*. Devel Immunol 1997;5:133–44.
[69] Flajnik MF, Kaufman JF, Hsu E, Manes M, Parisot R, Du Pasquier L. Major histocompatibility complex-encoded class I molecules are absent in immunologically competent *Xenopus* before metamorphosis. J Immunol 1986;137:3891–9.
[70] Hsu E, Du Pasquier L. Ontogeny of the immune system in *Xenopus*. I. Larval immune response. Differentiation 1984;28:109–15.
[71] Hsu E, Du Pasquier L. Ontogeny of the immune system in *Xenopus*. II. Antibody repertoire differences between larvae and adults. Differentiation 1984;28:116–22.
[72] DiMarzo SJ, Cohen N. An in vivo study of the ontogeny of alloreactivity in the frog, *Xenopus laevis*. Immunology 1982;45:39–48.
[73] Du Pasquier L, Flajnik MF. Expression of MHC class II antigens during *Xenopus* development. Devel Immunol 1990;1:85–95.
[74] Rollins-Smith LA, Blair P. Expression of class II major histocompatibility complex antigens on adult T cells in

Xenopus is metamorphosis dependent. Devel Immunol 1990;1:97–104.

[75] Ruben LN, Buchholz DR, Ahmadi P, Johnson RO, Clothier RH, Shiigi S. Apoptosis in the thymus of adult *Xenopus laevis*. Dev Comp Immunol 1994;18:231–8.

[76] Rollins-Smith LA, Barker KS, Davis AT. Involvement of glucocorticoids in the reorganization of the amphibian immune system at metamorphosis. Dev Immunol 1997;5:145–52.

[77] Barker KS, Davis AT, Li B, Rollins-Smith LA. In vitro studies of spontaneous and corticosteroid-induced apoptosis of lymphocyte populations from metamorphosing frogs/RU486 inhibition. Brain Behav Immun 1997;11:119–31.

[78] Denver RJ, Licht P. Neuropeptide stimulation of thyrotropin secretion in the larval bullfrog: evidence for a common neuroregulator of thyroid and interrenal activity in metamorphosis. J Exp Zool 1989;252:101–4.

[79] Denver RJ. Acceleration of anuran amphibian metamorphosis by corticotropin-releasing hormone-like peptides. Gen Comp Endocrinol 1993;91:38–51.

[80] Denver RJ. Environmental stress as a developmental cue: corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. Horm Behav 1997;31:169–79.

[81] Newman RA. Effects of changing density and food level on metamorphosis of a desert amphibian, *Scaphiopus couchii*. Ecology 1994;75:1085–96.

[82] Kupferberg SJ, Marks JC, Power ME. Effects of variation in natural algal and detrital diets on larval anuran (*Hyla regilla*) life-history traits. Copeia 1994;446–57.

[83] Martinez IP, Alvarez R, Paz-Herraez M. Growth and metamorphosis of *Rana perezi* larvae in culture: effects of larval density. Aquaculture 1996;142:163–70.

[84] Skelly DK, Werner EE. Behavioral and life historical responses of larval American toads to an odonate predator. Ecology 1990;71:2313–22.

[85] Rollins-Smith LA, Parsons SCV, Cohen N. Effects of thyroxine-driven precocious metamorphosis on maturation of adult-type allograft rejection responses in early thyroidectomized frogs. Differentiation 1988;37:180–5.

[86] Clem LS, Miller NW, Bly JE. Evolution of lymphocyte subpopulations, their interactions, and temperature sensitivities. In: Warr GW, Cohen N, editors. Phylogenesis of Immune Functions. Boca Raton, Fl: CRC Press, 1991. p. 191–210.

[87] Cooper EL, Wright RK, Klempau AE, Smith CT. Hibernation alters the frogs immune system. Cryobiology 1992;29:616–31.

[88] Miodonski A, Bigaj J, Mika J, Plytycz B. Season-specific thymic architecture in the frog *Rana temporaria*: SEM studies. Dev Comp Immunol 1996;20:129–37.

[89] Jozkowicz A, Plytycz B. Temperature but not season affects the transplantation immunity of anuran amphibians. J Exp Zool 1998;281:58–64.

[90] Maniero GD, Carey C. Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. J Comp Physiol B 1997;167:256–63.

[91] Diaz HF, Graham NE. Recent changes in tropical freezing heights and the role of sea surface temperatures. Nature 1996;383:152–5.

[92] Ader R, Felten D, Cohen N. Interactions between the brain and the immune system. Ann Rev Pharmacol Toxicol 1990;30:561–602.

[93] Ader R, Cohen N. Psychneuroimmunology: conditioning and stress. Ann Rev Psychol 1993;44:53–85.

[94] Parsons PA. Environmental stresses and conservation of natural populations. Annu Rev Ecol Syst 1989;20:29–49.

[95] Pechmann JHK, Scott DE, Semlitsch RD, Caldwell JP, Vitt LJ, Gibbons JW. Declining amphibian populations: the problem of separating human impacts from natural fluctuations. Science 1991;253:892–5.

[96] Karp JD, Moynihan JA, Ader R. Psychosocial influences on immune responses to HSV-1 infection in BALB/c mice. Brain Behav Immun 1997;11:47–62.

[97] Carey C. Factors affecting body temperatures of toads. Oecologia 1978;35:197–219.

[98] Vermeer BJ, Hurks M. The clinical relevance of immunosuppression by UV irradiation. J Photochem Photobiol 1994;24:149–54.

[99] Selgrade MJ, Repacholi MH, Koren HS. Ultraviolet radiation-induced immune modulation: potential consequences for infectious, allergic and autoimmune disease. Environ Health Perspect 1997;105:332–4.

[100] Norval M, El-Ghorr A, Garrssen J, van Loveren H. The effects of ultraviolet light irradiation on viral infections. Brit J Dermatol 1994;130:693–700.

[101] Rivas JM, Ullrich SE. The role of IL-4, IL-10 and TNF-α in the immune suppression induced by ultraviolet radiation. J Leucocyte Biol 1994;56:769–75.

[102] Grijzenhout MA, Aarts-Riemens MI, Claas FH, van Prooijen HC. Prevention of MHC-alloimmunization by UV-B irradiation in a murine model: effects of UV dose and number of transfused cells. Brit J Haematol 1994;87:598–604.

[103] Sharma RP, Reddy V. Toxic effects of chemicals on the immune system. In: Haey TJ, Berndt WO, editors. Toxicology. Washington: Hemisphere Publishing Corp, 1987. p. 555–91.

[104] Wester PW, Vethaak AD, van Muiswinkel WB. Fish as biomarkers in immunotoxicology. Toxicology 1994;86:213–32.

[105] Rice CD, Banes MM, Ardelt TC. Immunotoxicity in channel catfish, *Ictalurus punctatus*, following acute exposure to tributyltin. Archiv Environ Contam Toxicol 1995;28:464–70.

[106] Zelikoff JT, Bowser D, Squibb KS, Frenkel K. Immunotoxicity of low level cadmium exposure in fish: an alternative animal model for immunotoxicological studies. J Toxicol Environ Health 1995;45:235–48.

[107] Hart LJ, Smith SA, Robertson J, Holladay SD.

Exposure of tilapian fish to the pesticide lindane results in hypocellularity of the primary hematopoietic organ (pronephros) and the spleen without altering activity of phagocytic cells in these organs. Toxicology 1997;118:211–21.

[108] Devillers J, Extrayat JM, editors. Ecotoxicity of Chemicals to Amphibians. Philadelphia: Gordon and Breach Science Publishers, 1992.

[109] Baxter GT, Stromberg MR, Dodd CK. The status of the Wyoming toad (*Bufo hemiophyrs baxteri*). Environ Cons 1982;9:348.