CHAPTER 8

Evaluating the Impact of Pesticides in Amphibian Declines

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I. INTRODUCTION TO THE PROBLEM

In the global ecosystem, elements move through the earth's crust, bodies of water, atmosphere, and plant and animal life via the food web and circulation of air and water. It is then likely, if not inevitable, that the consequences of application of pesticides result in global distribution and dispersion of potentially toxic compounds through these same natural processes; these toxins could compromise plant and animal life across habitats. For instance, organisms in remote arctic marine ecosystems have been found to accumulate high levels of contaminants like PCBs (reviewed by Bard 1999) far from the sites of initial pollution; such results indicate that the site of contamination is not fixed and that contamination can gradually diffuse throughout the ecosystem. Many of the early synthetic organochlorine pesticides still persist in the environment because they are stable and biomagnify in the food web. Although many of these pesticides were banned in the United States and Europe in the 1970s (Peterle 1991), many continue to be used in developing nations.

New-generation pesticides break down quickly and are often thought to be less harmful than early synthetic pesticides, although they can also be present chronically at low-levels and be transported aerially, in runoff, and through above-ground and below-ground water sources (e.g., Thurman and Cromwell 2000). Short-term exposure to short-lived contaminants can also, therefore, exert chronic effects on organisms and may contribute to population declines (Cowman and Mazanti 2000). For these reasons, exposure to pesticides is repeatedly presented as a potential contributor to amphibian population declines and a threat to biodiversity in general (Corn 1994; Semlitsch 2003). The role that contaminants may play in declines has been addressed now in a number of volumes (including Sparling *et al.* 2000; Linder *et al.* 2003; Semlitsch 2003; Stuart *et al.* 2004; Lannoo 2005). Amphibian

declines continue to be a concern, particularly in areas where population density has decreased at the landscape level and where mass die-offs have occurred. It is not clear if populations that have not experienced declines are free from danger, if trouble has not yet reached them, or if they have already adapted to the hazard. Understanding how the major threats of habitat destruction and alteration, disease and pathogens, introduced species, global change, and contamination may singly and interactively influence populations is essential to pinpoint sources of danger and to construct solutions that remedy or reduce the impact. To address the question of how pesticides may contribute to the risk of amphibian population declines, this chapter evaluates (1) why linking declines with pesticides may be difficult, (2) the role pesticides may play in amphibian populations, (3) the approaches and results of studying declines, and (4) a strategy for assessing the causation and effects of contaminants in natural systems.

II. WHY LINKING DECLINES WITH PESTICIDES IS DIFFICULT

When Rachel Carson published "Silent Spring" in 1962, she was able to pull together large amounts of anecdotal and quantitative data to convincingly demonstrating that a number of chemical toxicants were negatively affecting organisms across taxonomic groups. So, then, if chemical contaminants are in fact linked to amphibian declines, should one not be able to make this connection relatively easily for a single taxonomic group? Or, is it possible that even if contamination by pesticides is a significant contributor to amphibian declines, making these links could still be difficult?

Certainly many of the pesticides in use today have some advantages over the early synthetic pesticides: they are less likely to bioaccumulate or magnify, they are less persistent in the environment, and federal regulations often maintain them at concentrations below limits lethal to non-targeted wildlife. Regulations banning the use of the early synthetic pesticides were meant to protect humans and non-targeted wildlife from the ill effects of contaminants, so those used presently should be "more benign," although reports of present-day chemicals disrupting endocrine system function make this proposal questionable (see review by Colburn *et al.* [1996]). Chemical effects on organisms, therefore, should be expected to be more subtle. Yet, chemicals like DDT also had subtle effects on bird reproduction, and scientists were still able to make cause-and-effect linkages. While it is believed that the role of pesticides in amphibian declines may be determined through experimental and correlational studies, there are a number of reasons why making the link between pesticides and amphibian declines in nature may be difficult to achieve.

Species differ in their sensitivity to pesticides. In initial discussions addressing whether declines were occurring, or if declines were just the results of natural variation in time and space (Pechmann *et al.* 1991; Pechmann and Wilbur 1994), researchers pointed out that while one species in a community may go extinct, other species appear to be unaffected or actually increase in abundance. Research has demonstrated variation within (Bridges and Semlitsch 2001) and among (Bridges and Semlitsch 2000) amphibian species in sensitivity to lethal levels of contamination, which supports observations in nature that some amphibian populations could decline due to pesticides (and/or other factors) while others in the same community appear to be unaffected. Linking declines with pesticides may be difficult because species are differentially affected by pesticides (as well as other stressors) and because a community is unlikely to have a unanimous response to stressors.

While differences in species' responses to pesticides may make declines difficult to detect, variation in pesticide concentrations and application rates, and the types of pesticides used fluctuate spatially and temporally, which incorporates another component of variation into the equation. Farmers routinely rotate their crops, which leads to changes in the kinds or amounts of pesticides used on a particular parcel of land. The amount of pesticide that drifts into nearby habitats or that runs off into aquatic habitats will also fluctuate with differences in seasonal weather conditions, such as the amount of wind or rain — even if pesticide application rates are consistent. Furthermore, a chemical's persistence will also

vary depending on environmental temperatures as well as amount of rainfall in a given year. This means that amphibians living in areas characterized by periodic use of pesticides may experience pulses of exposure in varied contexts. Making clear links between any particular pesticide and any exposure scenario (e.g., timing of exposure, interval of exposure, environmental conditions) with amphibian declines may therefore be extremely difficult based solely on field observations, because field conditions will almost certainly vary every year.

It is also conceivable that while pesticide exposure may play a role in declines, the impact may be tied to the presence of other stressors. It is often difficult to ascertain whether population declines are due to compounds applied to crops, or to concomitant ecological alterations such as habitat fragmentation or diversion of water. Many correlated factors must be considered when trying to determine the effects of pesticides on amphibians. Hecnar (1995) found that the amount of woodland surrounding a pond is the most important factor in determining species richness in southern Ontario ponds. Beja and Alcazar (2003) reported that the change from temporary bodies of water to permanent ones was the most important factor dictating amphibian presence or absence on agricultural lands. Knutson *et al.* (2004) found that ponds near grazed land and row crops experienced increases in turbidity, phosphorus, nitrogen, and low concentrations of agricultural chemicals. Factors such as presence or absence of fish, amount of vegetation, and area of the pond were more important in determining the presence of a species than were landscape variables. In southern Ontario, Bishop *et al.* (1999) examined species diversity and density, as well as hatching success and deformities, among marshes embedded in an agricultural matrix. They concluded that amphibians were more affected within agricultural areas than either upstream or downstream of agricultural lands; they suggested nutrient run-off to be the culpable agent. Bonin *et al.* (1997) also pointed out the difficulties in distinguishing between other agricultural practices and the application of chemicals to crops as the cause of decline of amphibian populations.

Determining critical interactions between multiple stressors will take time, and the presence of multiple factors increases the difficulty of directly tying declines to pesticides. Current research and thought suggest that multiple stressors may be the likely cause of amphibian declines (Carey *et al.* 2001; Linder *et al.* 2003), and there is some evidence of synergistic interactions among factors (Little *et al.* 2000; Relyea and Mills 2001; Boone *et al.* 2004; Bridges-Britton *et al.*, in review). Although post-application levels may be high enough to induce mortality in cases of direct exposure, more often pesticides will be transported aerially or in runoff at sublethal levels (LeNoir *et al.* 1999) so that direct lethal effects would not be anticipated. Even low levels of contaminants, however, could contribute to declines through interactions with other stressors.

Contaminants are also able to select for resistant genotypes that can disguise the role pesticides may be having. Large-scale monocultures now dominate many landscapes where diverse amphibian populations formerly thrived (Burnett 1997; Kupferberg 1997), perhaps due to selection favoring species more tolerant of stress and eliminating those that did not develop resistance. Meanwhile, amphibians that have the genetic ability to adapt to chemical contamination may still be hindered by agriculturally induced fragmentation of their habitats. Furthermore, populations often are not completely isolated from each other but rather are part of a network of populations known as a metapopulation. Among populations within a metapopulation, gene flow can ameliorate the net effect of a stressor on one of the populations. Semlitsch (2000) pointed out that two primary factors govern the dynamics of amphibian metapopulations: (1) the number and density of amphibians moving among ponds and (2) the probability of individuals reaching ponds. Thus, a reduction in number of ponds, the inimical nature of the terrestrial habitat surrounding ponds, and chemical contamination of ponds (which may make some ponds "sinks" [Rowe *et al.* 2001]) are all factors likely to be contributing to the demise of amphibians within an agricultural setting.

Other stressors, either natural or anthropogenic, may make it difficult to distinguish subtle effects of contaminants on metamorphosis. Small size of metamorphs, for example,

can result either from exposure to contaminants or from high densities of larvae. Therefore, although one knows (in general) that herbicides reduce body mass of anurans at metamorphosis, finding small metamorphs in agricultural areas in ponds known or suspected to be contaminated is not particularly informative, given this could result either from exposure to contaminants or from high larval densities. It is important to demonstrate the general patterns of responses experimentally and to know how particular pesticides or classes of pesticides alter those responses. One can then use an understanding of how the principle components of the community (amphibians, food resources, predators) are affected by contaminants to predict the consequences of contaminants in the field over time. Furthermore, knowing how various natural and anthropogenic factors interact with contaminants is requisite for evaluating how these factors intensify or reduce the contaminant's effect. Relyea and Mills (2001) found that treefrogs (*Hyla versicolor*) reared in the laboratory were significantly more affected by an interaction of predators and contaminants than by either alone and Boone and Semlitsch (2001, 2002) showed that manipulations of competition, predation, and the drying of ponds influenced the magnitude of the chemical effect. Bridges-Britton *et al.* (in review) demonstrated that susceptibility to a pesticide was greater in the presence of *Saprolegnia* or under ultraviolet radiation.

Relating declines in the wild to pesticides may be difficult for all the reasons listed above, and there seems to be no experimentation in progress designed to clarify this link. While laboratory and field mesocosm studies and correlational studies are all important for understanding the effects of pesticides on individual species, and on population and community dynamics, there are some critical pieces missing. Demonstration that contaminants affect hatching success, length of larval period, survival to metamorphosis and body-size at metamorphosis are important, but do not definitively reveal how population persistence will be affected over time. For example, unless a pesticide (or an interaction of a pesticide with another factor) completely and consistently eliminates a species through reproductive failure (poor hatching success or low survival to metamorphosis), current data are insufficient to make accurate predictions about the population trajectory through time. While reduced hatching success or reduced survival to metamorphosis seem like negative impacts (and they may well be), alone they do not indicate what the long-term impact on the population will be as long as some individuals continue to reach metamorphosis and maintain the population (Vonesh and De la Cruz 2002). Many species of amphibian may adapt to periodic bouts of reproductive failure. If exposure to pesticide, therefore, varies in time and space in ways that influence population dynamics, then the outcome will be confounded by natural variation in the population, making precise assessment of the impact of the contaminant difficult.

III. HOW PESTICIDES ELICIT AN EFFECT

One of the difficulties in evaluating the effects of pesticides on amphibians is that the response can vary from no apparent effect on one hand to mortality resulting in population declines and extinction on the other. Occasionally, amphibian populations have been shown to be reduced or eliminated locally as a result of mortality arising either directly or indirectly from contaminants such as heavy metals (Beyer *et al.* 1985), pesticides (Lambert 1997), and coal ash (Rowe *et al.* 2001). It is, however, exceptional that die-offs can be linked clearly to a particular contaminant or pesticide (especially at expected field application rates). The more subtle effects of pesticides are likely to be more common, yet are more difficult to diagnose or recognize in the field.

Subtle chemical effects may take a number of forms. Even when environmental levels of pesticides are below concentrations that would induce mass mortality, they may still have an impact on the persistence of a population through changes in body-mass at metamorphosis or time to metamorphosis. Pesticides may also have other sublethal effects, such as endocrine disruption (discussed elsewhere in this volume), altered growth rates of metamorphosed animals, and changes in critical behaviours. Time to metamorphosis and body-size at metamorphosis often are used in toxicological studies because they have been correlated

with fitness. Pesticides that can influence either of these have the potential to alter amphibian communities. Both of these traits are plastic, meaning that amphibians can trade early time to metamorphosis for smaller size at metamorphosis, or conversely, later time to metamorphosis for larger size at metamorphosis. In other words, these traits are inversely related to one another for many amphibian species. Mark-recapture studies of wood frogs (*Rana sylvatica*), mole salamanders (*Ambystoma talpoideum*), marbled salamanders (*Ambystoma opacum*), and chorus frogs (*Pseudacris triseriata*) have shown that differences in amphibian size at metamorphosis and time to metamorphosis can influence individuals throughout their lifetimes by affecting reproductive success, survival of post-metamorphic animals and lifetime size (i.e., animals that are larger than others at metamorphosis maintain this size-advantage throughout their lifetime) (Smith 1987; Semlitsch *et al.* 1988; Berven 1990; Scott 1994). Werner (1986) suggested, however, that species might have different strategies for utilizing aquatic and terrestrial environments. Some experimental studies have demonstrated that some species may be able to compensate for small size at metamorphosis (e.g., American toads; Goater [1994], Boone [2005]). In species with short larval periods, such as the American toad, early metamorphosis may foster greater growth of terrestrial stages prior to winter. It may be necessary to take into account both time to metamorphosis and size at metamorphosis in order predict persistence of populations adequately.

Even if exposure to pesticides results in reduced mass (without any compensation in the length of the larval period), population density might not change initially. If smaller metamorphs are less likely, therefore, to survive the winter (as evidence suggests) then over a number of years population size may dwindle to extinction. In natural systems, roughly 3–5% of amphibian eggs reach metamorphosis (Semlitsch 1987; Berven 1990; Semlitsch *et al.* 1996). Even if this value remains unchanged but individuals leaving the ponds are smaller, then the percentage of juveniles suffering winter mortality would increase (assuming exposure and response to exposure remains constant), even when the pesticide affects only the aquatic stages. As a population decreases in size it may become vulnerable to "small population effects" such as inbreeding depression (as found in fragmented populations of anurans; Hitchings and Beebee [1997]; Beebee [2001]) or increased risk of extinction due to stochastic events (Pechmann *et al.* 1989; Hels 2002), thereby further exacerbating the problem.

A pesticide may affect body-mass at metamorphosis, time to metamorphosis, or survival to metamorphosis, either directly by influencing individual physiology or behaviour, or indirectly by altering the food web. For instance, neurotoxic insecticides may affect amphibians directly by disruption of nervous system function. At high environmental levels this may prove lethal but at sub-lethal levels may influence critical swimming, feeding, and defensive behaviours (Berrill *et al.* 1993; Semlitsch *et al.* 1995; Bridges 1997; Eroschenko *et al.* 2002; Greulich and Pflugmacher 2003; Rohr *et al.* 2003) or predator-prey interactions (Bridges 1999; Ingermann *et al.* 2002). In ephemeral ponds, even short-term impairment of feeding behaviour may decrease the probability of an organism reaching metamorphosis before the pond dries. Lowering of the feeding rate likewise may reduce size at metamorphosis and/or lengthen the time to metamorphosis. Additionally, the breaking down of pesticides in the body incurs a metabolic cost in insects (Appel and Martin 1992; Bernard and Lagadic 1993), crayfish (Rowe *et al.* 2001) and amphibians (Rowe *et al.* 1998), which may reduce the amount of energy available for growth and development. There may also be important effects of pesticides on the terrestrial life stage of amphibians. Although the effects of contaminants on terrestrial stages of amphibians have been relatively unstudied, a few investigations have indicated that pesticides may have important effects on juvenile and adult anurans (Hopkins *et al.* 1998; Mann and Bidwell 1999; Gendron *et al.* 2003; James *et al.* 2004). Pesticides can disrupt mating behaviour so that breeding migrations are affected or reproductive success lowered, and, over time, populations decline (Park *et al.* 2001). Accumulated evidence suggests that pesticides have endocrine-disrupting properties (Hayes *et al.* 2002, 2003; Goulet and Hontella 2003; MacKenzie *et al.* 2003; Howe *et al.* 2004) that can directly affect development of testes, ovaries, and secondary sex characteristics (such as pharyngeal size), thereby impairing reproductive behaviours. Furthermore, James *et al.*

(2004) suggested that amphibians might be more susceptible to population reductions if exposed to pesticides when they are less mobile (i.e., hibernating in contaminated areas).

Pesticides can indirectly affect amphibians through changes in the food web. Concentrations of contaminants that cause no direct risk to an amphibian species may have serious impacts on its food resources and thus indirectly result in a population decline. Survival to metamorphosis in salamanders has been found to decline with exposure to insecticide, an effect attributed to decreases in their zooplanktonic prey (Boone and James 2003). Additionally, body-mass at metamorphosis can be reduced when herbicides reduce algal food resources (Diana *et al.* 2000; Boone and James 2003). Mills and Semlitsch (2004) experimentally demonstrated that, for the insecticide carbaryl, indirect effects on the food web had greater influence on amphibians than did direct chemical effects; this appears to be the only study explicitly testing such a relationship. Contaminants may also affect the efficacy or the survival of predators upon amphibians. Insects are significant predators on many larval amphibians (Wilbur and Fauth 1990) and are an important food resource for terrestrial amphibians. Insecticides are intended to be lethal to insect pests, and invertebrates in general will be particularly sensitive to insecticide exposure, which could increase abundance of larval amphibians thereby increasing competition (Boone and Semlitsch 2003; Relyea *et al.* 2005) and potentially reduce growth and/or survival of terrestrial amphibians. Elimination or reduction of predation could result in high larval survival so that individuals experience high competition for food resources, which in turn may result in death via starvation and reduced size of metamorphs (e.g., the ameliorative effects of predation [Morin 1981]).

Low exposure to chemicals may result in increased metabolic expenditure that could reduce the energy available for growth and reproduction while having no observable effects on the food web. Contaminants may also suppress function of the immune system thereby making organisms more susceptible to other environmental factors, both anthropogenic and natural (Carey 1993; Carey and Bryant 1995; Carey *et al.* 1999; Hayes *et al.* 2006). Sudden die-offs in Australia, Central America, western United States, and parts of Europe have been attributed to a chytrid fungus (Berger *et al.* 1998; Bosch *et al.* 2001; Muths *et al.* 2003), which may be a novel pathogen in these environments. The presence of chemicals in the environment, however, may make organisms more likely to succumb to pathogens or diseases that were already present (Parris and Baud 2004; Parris and Cornelius 2004; Bridges-Britton *et al.*, in review). Christin *et al.* (2004) found that exposure to mixtures of chemicals commonly found in agricultural settings had the potential to compromise immune system function, leaving individuals more susceptible to disease. Changes in individual immune responses or in metabolism may be markers of significance at the population level and could contribute to an understanding of how contaminants affect amphibians and influence sensitivity to other factors.

IV. APPROACHES TO STUDYING DECLINES

Although linking pesticides to amphibian declines may be difficult, there are a number of reasons why amphibians may be susceptible to pesticides and other chemical contaminants. Many researchers have suggested that amphibians may be particularly vulnerable to contaminated environments because they (1) have permeable eggs, skin, and gills that may increase absorption of toxins into the body, (2) often occupy critical aquatic and terrestrial habitats where their chances of encountering contaminants are high, (3) live in environments stressful for larvae where the addition of a pesticide may further reduce juvenile recruitment, (4) are generally philopatric to natal sites and thus unlikely to avoid contaminated sites, and (5) can hibernate, which may subject them to pesticides during times when they cannot avoid exposure (Henry 2000 and references therein). Some research suggests, however, that amphibians are not particularly sensitive to chemical contamination (Bridges *et al.* 2002), at least not more so than species of vertebrates typically used in

toxicological testing (i.e., fish). The simple fact that chemical contaminants are widespread, however, means that one should try to understand what role they are playing in amphibian populations and communities.

Recent research has focused on assessing the importance of multiple stressors in amphibian declines. Contaminants do not exist in nature in isolation from other environmental variables, and so it becomes necessary to construct more elaborate experimental designs to incorporate multiple factors. These may interact in a manner not predictable merely from an understanding of the effects of the individual factors. Environmental stressors can interact with one another in three ways. They can interact antagonistically, whereby the combined effect of the stressors is less than would be predicted from the sum of their individual effects. They can be additive, in which their collective effect is simply the sum of their individual effects. Finally they can be synergistic, whereby the effect is greater than would be predicted by simply adding the effects of the individual stressors. A multiple-stressor approach has been advocated in many studies that have examined single stressors, and current research suggests that synergistic interactions may contribute to declines (Carey *et al.* 2001; Linder *et al.* 2003 and references therein).

Researchers have conducted studies in the laboratory, in mesocosm field studies and at the landscape level to assess contaminant effects. Each of these research tiers will help to evaluate the potential outcome that exposure to pesticides will have in the field in the presence and absence of other factors, and will help to evaluate the mechanisms driving the effects in the field. Integrating the information collected in the laboratory and from studies at the level of the mesocosm, field and landscape will help to determine contaminant classes that pose the greatest risk and to ascertain which populations will most likely be affected. Some of the important concepts that research in amphibian toxicology has established are highlighted below.

A. Laboratory Studies

Laboratory studies represent a first-tier effort in understanding the effects of environmental contaminants on amphibians. It is in the laboratory that controlled efforts can determine how a particular compound affects an organism. It is necessary to observe singular effects of contaminants if one is to predict responses in a natural habitat. Compounds that kill insect pests by disrupting cholinesterase, for instance, may also have similar effects on non-target vertebrates, such as amphibians. Research in the laboratory has helped evaluate: (1) how changes in behaviours can influence a contaminant's effect, (2) the relative sensitivity within amphibians, and compared to other taxa, and (3) how contaminant influences sensitivity to other factors.

In the laboratory, traditional experimental studies in amphibian toxicology are usually aimed at understanding the physiological basis for the mode of action of a chemical and its effect on mortality, growth, development, and morphological deformities (e.g., LC_{50}s or Frog Embyro Teratogenesis Assay — *Xenopus*; American Society for Testing and Materials 1991). Laboratory studies have become more sophisticated than the "kill-them-and-count-them" tests conducted in early days. Because contaminants do not generally occur at environmental concentrations great enough to elicit direct mortality, traditional LC_{50} studies (determining the concentration of a compound required to kill 50% of a test population) are less useful today than studies examining the subtler effects of lower, sub-lethal concentrations. Examining behaviour, growth and development can provide a better understanding of the impact of low-level exposure of pesticides on amphibian life histories. Small changes in life-history characteristics can alter population dynamics (e.g., juvenile recruitment, fecundity) and cause declines in population numbers over time, making examination of sub-lethal endpoints particularly important.

Behavioural responses to chemicals are most easily measured in the laboratory, and represent important endpoints. Toxic compounds can cause indirect mortality and

detrimental changes in life history by altering such behaviours as swimming, feeding, or predator avoidance (Little *et al.* 1990). The presence of an environmental contaminant, for instance, can decrease (Freda and Taylor 1992; Bridges 1997) or increase (Rohr *et al.* 2003) larval amphibian activity and alter swimming and feeding behaviour (Freda and Taylor 1992; Bridges 1997). Skelly (1994) demonstrated that diminished activity generally leads to decreased predation by visually oriented predators. Alternatively, it is possible for contaminants to increase activity (Rohr *et al.* 2003) and, therefore, predation rates (Cooke 1971). Behavioural responses such as these are difficult to observe and control in the field, but may explain the mechanisms through which pesticides affect populations.

In the laboratory it is also possible to determine how amphibians respond to chemicals relative to other, more commonly used, test species such as rainbow trout, bluegill sunfish, or fathead minnows that are used by governmental agencies to set environmentally acceptable limits. For example, studies have suggested that allowable levels of copper in the environment, as determined by tests using a fish species, may not be protective of amphibians (Bridges *et al.* 2002). Copper is introduced into the environment through mining and industrial practices and is used as an algaecide. Application of this metal can thus potentially contaminate amphibian habitats, such as ponds, and be injurious to amphibian larvae. Conversely, amphibians are not as sensitive to some pesticides (e.g., permethrin, carbaryl) as are other taxa (Bridges *et al.* 2002).

Laboratory studies are useful in that they allow researchers to understand how single pesticides affect species in the absence of other factors, so that differential sensitivity of species and life stages can be determined (Bridges 2000). This is important when considering how pesticides may influence the evolution of resistance to increasingly contaminated environments. Bridges and Semlitsch (2001) found significant differences in responses to carbaryl exposure among half-sibling families within a population of southern leopard frogs (*Rana sphenocephala*), indicating the presence of additive genetic variance (i.e., heritability) and likely, the ability to adapt to environmental stressors like carbaryl. Additionally, because responses to pesticides are partly genetic, selection for resistant individuals can occur. Semlitsch *et al.* (2000) found that treefrog (*Hyla versicolor*) tadpoles from clutches most sensitive to high levels of carbaryl (as determined in the laboratory) had lower survival under some field conditions (i.e., high density), indicating that resistance may come at the price of reduced competitiveness in natural, "clean" habitats. Furthermore, the timing of exposure influences the effect of a contaminant (Bridges 2000; Greulich and Pflugmacher 2003), which suggests that breeding phenology and management of pesticide application could be co-ordinated to minimize negative effects on amphibian populations.

Laboratory studies indicate that the presence of sub-lethal contaminants may be more potent in the presence of other factors. De Solla *et al.* (2002) found that developing tadpoles *in situ* had reduced hatching success, lower survival, and more deformities than did individuals reared in the laboratory in water from the same site. This suggests that some factor or factors present in the natural environment influence the toxicity of the compounds present there. Another example involved the photo-enhanced toxicity of a chemical by ultraviolet-B radiation (Zaga *et al.* 1998; Chapter 6 in the present volume). The results of this two-factor design indicate that ultraviolet-B and the insecticide carbaryl each affected tadpole responses separately, but that in combination the toxicity of carbaryl increased tenfold above that when the tadpoles were subjected to either factor alone. Additionally, higher water temperature increased mortality of *Rana clamitans* tadpoles at four of the seven highest concentrations of carbaryl (Boone and Bridges 1999), suggesting that natural stressors can influence the potency of contaminants. When tadpoles were reared with carbaryl and non-lethal (caged) predatory salamander larvae, they had higher mortality than when reared with predators or carbaryl alone (Relyea and Mills 2001). Observing such interactions in the laboratory provides a better understanding of responses in the field, and yields predictions for changes in natural populations.

Another example of uncovering responses that are unpredictable from examining singular effects is a study of two natural Minnesotan ponds (one reference and one

chemically impacted), each tested in the presence and in the absence of ultraviolet radiation (Bridges *et al.* 2004). The incidence of embryonic deformities was high in the reference site when ultraviolet radiation was excluded, but low when ultraviolet radiation was present. This suggests the presence of a permanent, naturally occurring, deleterious compound that is broken down by ultraviolet radiation. In the impacted site, deformities were greatest when ultraviolet radiation was present, indicating the presence of a compound(s) potentiated by ultraviolet radiation. Ultraviolet radiation is present in nature and accordingly it can influence the toxicity of some contaminants. This highlights the importance of amalgamating laboratory and field studies to achieve a clear understanding of the impacts of contaminants in natural environments.

B. Mesocosm Field Studies

Toxicological studies in the laboratory are critical for determining lethal ranges of contaminants for a large number of species and to understand mechanisms that might drive effects seen in nature. Field studies are often viewed as being more time-consuming, more difficult to assess, and more variable. While to some extent these views may be valid, field studies are necessary for determining how contaminants will affect populations and communities in more complex environments. Mesocosm studies are ideal for combining the benefits of both field and laboratory studies and have been used to examine the role of pesticides in amphibian declines (Rowe and Dunson 1994; Boone and James 2005). Boone *et al.* (2004) found that large-scale experimental ponds yield similar results to mesocosm studies conducted in cattle-tank ponds, suggesting that mesocosm studies are a powerful tool for evaluating field-level responses of populations and communities. From ecotoxicological studies conducted in mesocosms, it has been learned that (1) environmental conditions in ponds to which pesticides are added can influence the magnitude of the chemicals' effects, (2) indirect effects of other factors on the food web can dramatically alter the response to the contaminant, (3) laboratory studies are often not predictive of field-level responses, and (4) expected environmental concentrations of pesticides could contribute to some amphibian declines.

Mesocosm studies have contributed to an understanding of how contaminants may influence populations in complex natural environments, and also how diversity may be influenced by contaminants. When factors, such as larval density, predation, and pond-drying, that are known to be important to natural amphibian communities (Semlitsch *et al.* 1996) are manipulated with chemical contaminants, natural stressors can influence the magnitude of the chemical effect (reviewed by Boone and Bridges [2003]). Furthermore, the addition of multiple factors is hypothesized to contribute to amphibian declines and exacerbate the chemical effect, a result also supported by laboratory studies (see above). For instance, amphibians appear to be more susceptible to trematode infection in the presence of pesticides such as atrazine, malathion and esfenvalerate (Kiesecker 2002). Additionally, there is some evidence that indicates multiple stressors have greater effects than do single factors alone (e.g., Britson and Threlkeld 2000; Gendron *et al.* 2003; Hatch and Blaustein 2003; Boone *et al.* 2005; Boone *et al.*, in press). For instance, Bridges-Britton *et al.* (in review) demonstrated that the effects of the insecticide carbaryl became more lethal when both ultraviolet radiation-B and a waterborne pathogen (*Saprolegnia ferax*) were present than when just one or two factors were present in a laboratory environment. In a field study conducted simultaneously, they found that the presence of both the pathogen and expected environmental concentrations of carbaryl decreased survival for southern leopard frogs, suggesting that the accumulation of stress under more realistic conditions can have negative consequences for the population. Thus, there was a congruence of laboratory and field studies.

Another important conclusion drawn from mesocosm studies is that contaminants can affect amphibian population and community dynamics by modifying the food web. Pesticides and other contaminants often have potent effects on larval amphibians' food base (algae or zooplankton), as well as on their predators, especially aquatic invertebrate ones such as

insects and crayfish. Most expected environmental concentrations of pesticides appear to be below a level needed to induce direct mortality, but these concentrations can increase or decrease food resources. Herbicides can reduce the amount of algal resources upon which anuran tadpoles feed (Diana *et al.* 2000; Boone and James 2003), while insecticides can reduce the abundance of (or eliminate) zooplankton on which carnivorous salamander larvae prey (Hanazato and Yasuno 1990; Boone and James 2003). In this way, one can predict survival, time of metamorphosis and size at metamorphosis by knowing what parts of the food web are most affected by a pesticide (Fig. 1). For instance, the insecticide carbaryl predictably results in trophic cascades. Carbaryl lowers the abundance of zooplankton and predatory invertebrates, which results in an algal bloom. As a result of carbaryl exposure, primary consumers increase (Boone and Semlitsch 2002) and secondary consumers decrease in abundance (Boone and James 2003). Leftcort *et al.* (1999) examined tadpole and snail communities exposed to heavy metals and predator cues and found that the metals shifted the outcome of competitive interactions and altered species abundance. An empirical measure of indirect and direct chemical effects was conducted by Mills and Semlitsch (2004) with the insecticide carbaryl, indicating that the indirect effect of carbaryl outweighed any direct effects through its effects on the food resources.

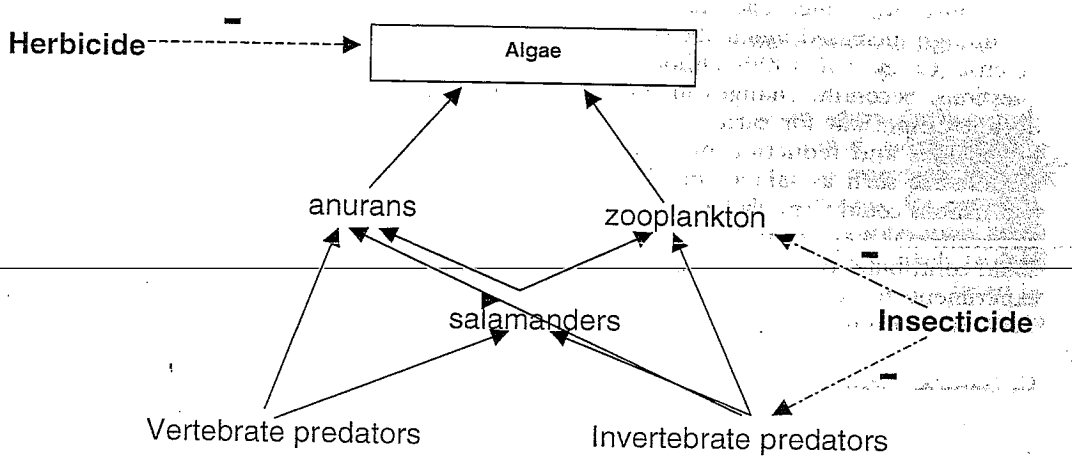

Fig. 1. Diagrammatic model of the effect of herbicides and insecticides on aquatic communities containing amphibians. The model suggests that in general (1) herbicides that reduce the algal food base of a community will reduce the abundance of all species in the food web and have negative impacts on anuran and salamander survival, mass, and time to metamorphosis and that (2) insecticides that have sublethal effects on amphibians can negatively affect all invertebrates in the community and result in trophic cascades that can positively affect anurans (through increasing food resources) and negatively impact salamanders (through decreased food resources).

Another interesting pattern arising from mesocosm studies is that they suggest laboratory studies may not always accurately reflect field responses or predict population and community-level consequences of exposure to contaminants. This does not preclude laboratory studies usefully addressing other issues, such as understanding underlying mechanisms. Laboratory studies on contaminants can result in overestimating the effect found in the field, while missing other important effects. Relyea and Mills (2001) and Relyea (2003), for instance, found that tadpole mortality increases significantly for some anuran species in the presence of pesticides and predators combined over mortality caused by either factor alone. This suggests that in complex environments even low levels of contamination, if present in conjunction with predators (which is almost always the case), may lead to increased mortality. Field studies, however, suggest that these interactions may not always be significant in the field because other factors (like changes in food resource-base) may be more important (Boone and Semlitsch 2001; Boone and Semlitsch 2003; Mills and

Semlitsch 2004). Field studies including both predators and pesticides have not demonstrated synergistic interactions resulting in greater mortality. Additionally, laboratory studies have found that contaminants may have photo-enhanced toxicity with exposure to environmental levels of ultraviolet radiation (Zaga *et al.* 1998), which suggests that lethal concentrations may be much lower in nature than laboratory studies suggest. Field studies, however, have not shown any negative effects of exposure to sub-lethal levels of contaminants in the presence or absence of ultraviolet-B radiation (Bridges and Boone 2003), presumably because tannins in the water column can effectively filter out ultraviolet light and reduce the potential for phototoxicity.

Mesocosm studies do suggest that pesticides could contribute to population declines in two ways. First, for communities exposed to any anthropogenic stressors (which are *additional* stresses), the likelihood of declines may increase; species at the edge of their ranges or those near their physiological limits (in terms of temperature, pH, or other factors) may be the most susceptible to contaminant stress. Evidence suggesting that amphibians are more susceptible to pathogens (Parris and Cornelius 2004; Bridges-Britton *et al.*, in review), parasites (Gendron *et al.* 2003), ultraviolet radiation (Hatch and Blaustein 2003), and other contaminants (Boone *et al.* 2005; Hayes *et al.* 2006) in the presence of low-level contamination supports the notion that multiple sub-lethal stressors can reduce amphibian abundance over time. The mechanism whereby cumulative stressors affect organisms may be through increased metabolic costs for detoxification or through reduced immunological defense (Carey *et al.* 1999), although there has been very limited research addressing these questions. Secondly, changes in the food web may be an important contributor to some declines, especially for carnivorous amphibians. Invertebrates are sensitive to low levels of insecticides and reduction or elimination of them will have an impact on carnivorous amphibians such as salamander larvae. Boone and James (2003) found that salamander populations could virtually be eliminated from aquatic environments by exposure to carbaryl. Experimental mesocosm data do suggest that exposure to sub-lethal levels of contaminants could contribute to the problem of declining populations; linking patterns observed in such experiments to declines in the field would help determine the degree to which contaminant stress is important in the natural world.

C. Natural Field Studies

Ultimately, only natural field studies can link pesticides with actual declines of amphibian populations and provide powerful associations between a conjectural cause and effect. *In situ* field studies provide a means of examining broad, landscape-level responses to a factor, and of ascertaining whether aerial, or other, transport of pesticides is important. Such studies should lead to the emergence of landscape-level patterns. Most field research has focused primarily on three aspects: (1) measuring the accumulation of pesticides in individual amphibians across the landscape, (2) physiological responses to exposure, and (3) landscape-level patterns of declines. So to date, what do natural field studies reveal about the possible role of pesticides in enigmatic declines — that is, declines that cannot be explained by simple habitat destruction? The most basic tenet that field studies have revealed is that declines have occurred in "pristine" areas. There are, therefore, population declines that cannot be explained either by gross alteration of habitat or by introduced predators. This finding indicates that some factor or factors, such as disease, ultraviolet-B radiation, or contaminants must be playing a role in enigmatic declines. This is an important starting point. If one could explain declines on the basis of habitat destruction or exotics there would be no need to investigate other factors that are much more difficult to study.

Worldwide, amphibian declines appear to be most severe in montane areas, which generally are not subject to runoff from agricultural land. While these locations provide protection from the high concentrations of pesticides found in agricultural runoff waters, aerial transport can bring pesticides to even the most remote areas. For example, atmospheric transport carried the pesticide toxaphene from southern United States to the Great Lakes region (Ryan and Hites 2002) and even to the Arctic (Derek *et al.* 1990).

Although long-range pesticide transport is possible, transport over medium ranges may be more relevant because it can result in deposition of higher concentrations of pesticides. This is especially true for pesticides that break down rapidly and do not bioaccumulate. In California, pesticide transport from the Central Valley into the Sierra Nevada at distances up to 150 km is well documented (Zabik and Seiber 1993; Aston and Seiber 1997; McConnell *et al.* 1998; LeNoir *et al.* 1999). In fact, Bridges and Little (2005) report that tadpoles took longer to reach metamorphosis when exposed to extracts from air and water taken from an alpine site in Sequoia Kings Canyon Nationa Park in the Sierra Nevada Mountain range.

Three of the regions of the world with the best studied amphibian population declines, the Sierra Nevada of California, Western Australia, and the mountains of Central America, are all downwind from extensive agricultural areas. Lips and Donnelly (2005) pointed out that in Australia and Central America lowland populations that are much closer to agricultural regions (and are likely, therefore, to have greater exposure to pesticides) have not declined as much as have populations at higher elevations. This pattern suggests that if pesticides are contributing to declines, it is not through direct effects, but rather in synergism with some other factor (e.g., cold or disease) that is not present or active in the lowlands. A similar pattern exists in the United States. In the southern and southeastern regions there is extensive use of pesticides, but few amphibian declines, while in California there is heavy pesticide use and widespread amphibian population declines. Like the elevational pattern of declines in Australia and Central America, the pattern in the United States again suggests that pesticide exposure alone is not the sole cause of declines.

While it is clear that pesticides are transported into remote regions where declines have occurred, little is known about the resulting geographic and temporal distribution of pesticide residues impinging on amphibians. The temporal distribution is especially difficult to quantify because pesticide applications are extremely "spiky." In California, for example, pesticide applications during a few weeks of the year typically account for a substantial share of total use (Fig. 2). This means that field sampling of pesticides in water is likely to miss peak concentrations and, therefore, will underestimate short-term exposure levels faced by animals. In order to conduct experiments with ecologically relevant doses, one needs to know the levels to which animals are exposed in the field.

California has received the most extensive studies of pesticide transport to montane areas in which there are amphibian declines. No studies involving transport and deposition of pesticides into montane areas seem to have been carried out for Central America or Australia. Even in California, less than a dozen montane sites have been examined for residues relevant to amphibian declines. Furthermore, transport of only a few different chemicals, mainly endosulfan, chlorpyrifos, malathion, and diazinon, have received attention (Zabik and Seiber 1993; Aston and Seiber 1997; McConnell *et al.* 1998; LeNoir *et al.* 1999). Currently, however, many hundreds of different chemicals are in use. During 2000 in California alone, 781 different pesticides were used (Department of Pesticide Regulation 2001).

A growing number of studies in California are bypassing issues of transport and deposition; instead, researchers are seeking and finding pesticides directly in the bodies of frogs. As early as 1970, DDT residues were found in mountain yellow-legged frogs (*Rana muscosa*) throughout the Sierra Nevada (Cory *et al.* 1970). It was almost thirty years before the next study was completed when Datta *et al.* (1998) examined Pacific treefrogs (*Hyla regilla*) for DDE, chlorothalonil, and chlorpyrifos at two Sierra Nevadan sites. Sparling *et al.* (2001) also examined Pacific treefrogs and found higher pesticide levels in the Sierra Nevada, where a number of amphibian species have declined, than at two coastal sites that have experienced few declines. Pesticide residues in mountain yellow-legged frogs were compared between an experimental reintroduction site in the Californian Sierra Nevada where survival was very low and a site with healthy frog populations (Fellers *et al.* 2004). Of the six chemicals studied, five were found at higher levels in frogs from the reintroduction site (three significantly so) than in those from the comparison site. Finally, Angermann *et al.* (2002) found that although the pesticide toxaphene had been banned from use for

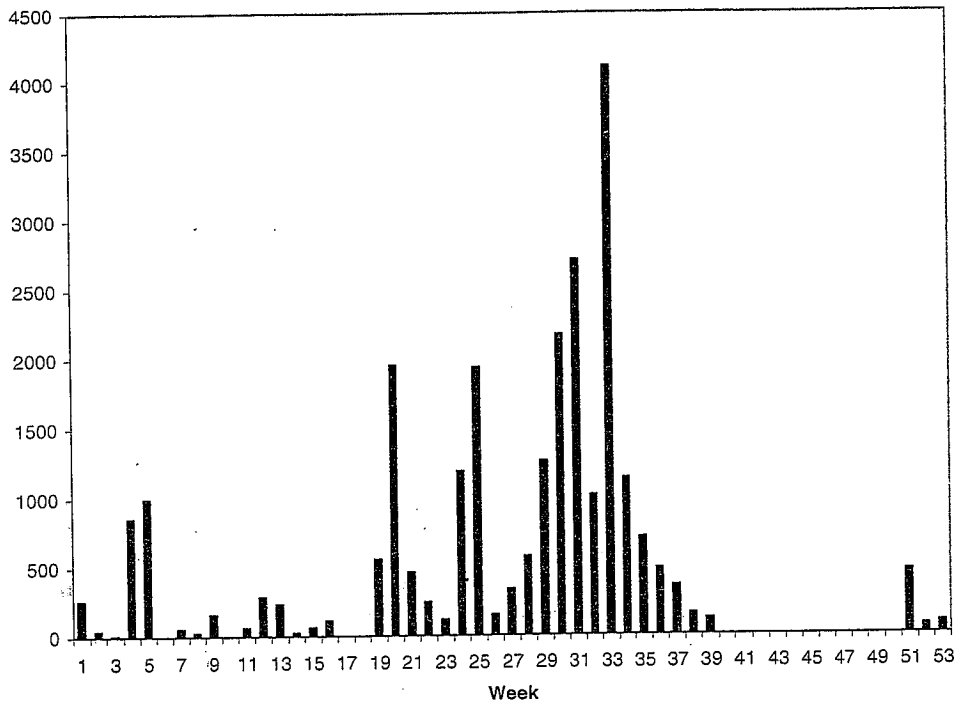

Fig. 2. Timing of chlorpyrifos pesticide applications upwind of historic sites for Cascades frogs (*Rana cascadae*) in northern California in 1998. Applications are kilograms of active ingredients. The locations of sites are from Jennings and Hayes (1994). Pesticide data are for 1998 (California Department of Pesticide Regulation; annual report on pesticide use [1999]). The methodology for calculating upwind use is described by Davidson (2004).

almost 20 years, it was still widespread in Pacific treefrogs from 21 sites across the Sierra Nevada.

It is clear that pesticides are widespread in the bodies of frogs even in rather remote regions. At this time, however, the biological significance of the levels of pesticides that have been found is still unknown. The situation is similar to that in humans; pesticide residues appear to be widespread in the bodies of people in the United States (CDC 2003) but the risks to health of the measured levels of residues are unknown. Understanding the biological effects of field-relevant doses of pesticides is a key challenge for future laboratory experiments and field observational studies.

To date, there are only a few studies directly indicating that exposure to sub-lethal levels in the field has biological effects. Sparling *et al.* (2001) found that pesticide residues were higher and cholinesterase levels lower in Pacific treefrogs in the Californian Sierra Nevada than in treefrogs on the coast. The low cholinesterase levels in Sierra Nevadan frogs may have resulted from exposure to cholinesterase-inhibiting pesticides (most organophosphate and carbamate pesticides). Without further research, however, one cannot rule out alternative explanations for suppressed cholinesterase levels, such as cold or altitude stress. Two studies have combined laboratory experiments with field observations or field experiments to demonstrate that exposures to pesticides in the field can suppress aspects of the anuran immune system. Gilbertson *et al.* (2003) found that leopard frogs (*Rana pipiens*) collected in Ontario in areas exposed to pesticides had lower antibody responses and cellular immune responses than did frogs from areas with less exposure to pesticides. In the laboratory, they found that sub-lethal levels of DDT, dieldrin, and malathion all suppressed the activity of the immune system of leopard frogs. Kiesecker (2002) studied the effects of exposure to

pesticides on wood frogs' (*Rana sylvatica*) susceptibility to trematode infection and found that frogs in sites exposed to agricultural runoff had dramatically greater trematode-induced deformities of the limbs than did frogs not exposed to pesticides (26% versus 4%). Companion laboratory experiments found that exposure to atrazine, malathion, and esfenvalerate all suppressed frogs' immune response and increased the success rate of trematodes encysting in frogs.

Some of the strongest evidence for a link between pesticides and actual amphibian population declines comes from studies by Davidson *et al.* (2001, 2002). They examined landscape-scale patterns of decline at almost 1 500 historic locations across California for eight amphibian species. For each site, they calculated the amount of upwind agricultural land-use based on predominate wind patterns, as a rough proxy for sites' exposure to pesticides. For four species of ranid frogs (*Rana aurora draytonii, R. boylii, R. cascadae* and *R. muscosa*), population declines were strongly associated with the amount of agricultural land use upwind from a site, suggesting that wind-borne pesticides may be an important factor in declines. In multivariate regression models, the association between declines and upwind agricultural land use was strong, even when other factors such as latitude, elevation, precipitation and local land-use were taken into account. It was striking that the same association held for four species with different ranges.

The studies by Davidson *et al.* (2001, 2002) did not contain actual data on pesticide use, and instead relied upon the amount of upwind agricultural land use as a proxy for actual pesticide use. A follow-up study, however, did find a strong association between declines for five species and upwind pesticide use, based on California state records of actual pesticide applications from 1974 to 1991 (Davidson 2004). In multivariate regression models upwind pesticide use was significant and a strong predictor of site-status for *Rana aurora draytonii, R. boylii, R. cascadae,* and *R. muscosa*. Furthermore, for these four species and the Yosemite toad (*Bufo canorus*) taken together, upwind use of cholinesterase-inhibiting pesticides was more strongly associated with declines than were total pesticides or any of 64 classes of pesticides (Davidson 2004).

Although the work by Davidson *et al.* (2001, 2002) and Davidson (2004) provided some of the best links between actual amphibian population declines and pesticide use, it has several limitations. First, although the association between population declines and upwind pesticide-use holds even when a number of covariates are taken into account, the pattern has not been tested in studies that also consider exotic predators or disease. Second, the strength of association between upwind pesticide-use, predominant wind patterns, and actual exposure levels are currently unknown. Ideally, one would want to know the actual exposures (e.g., concentration of residues in water or in frogs' bodies) for a large number of sites, but this information is not available. Clearly, a key area for future fieldwork is to measure exposure at a sufficiently large number of sites to be able to evaluate the relationship between the geographic pattern of exposure and patterns of species' decline.

Due to the large temporal and spatial scale of amphibian declines, they can never be directly subjected to experimentation. Therefore, the role of pesticides in amphibian population declines must be studied by a combination of large-scale observational studies, and manipulative laboratory and field experiments. If pesticides are affecting amphibian populations, it must be through a web of processes and relationships including the geographic patterns of agricultural land use, the pattern of pesticide use, and site-specific interactions with other factors. All of these processes ultimately contribute to large-scale patterns of amphibian population decline. The causal relationships generate patterns that can be called the "pesticide puzzle" of how pesticides may be affecting amphibian populations (Fig. 3). The various pieces in the puzzle (causal relationships and patterns) provide opportunities for many different types of studies, all of which contribute to understanding and evaluating the links between pesticides and amphibian declines.

Observational studies can examine many different parts of the pesticide puzzle. For example, Davidson *et al.* (2001, 2002) examined the association between the geography of

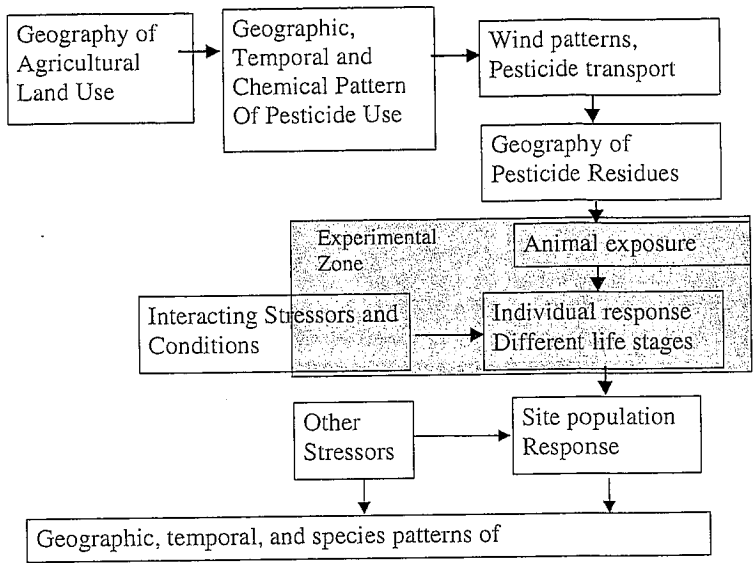

Fig. 3. Pieces in the pesticide puzzle: Diagram showing ways pesticides contribute to amphibian declines. All the relationships between the pieces in the puzzle present opportunities for research on the role of pesticides in declines. The gray box labelled "Experimental Zone" indicates relationships that are subject to experimental study. Only half the interacting stressors are included in the experimental zone to indicate that laboratory and mesocosm studies can never be sure of including all the important interactions in natural systems.

agricultural land use, wind patterns, other population stressors, and geographic patterns of decline. Ultimately, observational studies need to be coupled with experiments that can explore mechanisms at the level of individual animals. A variety of laboratory and mesocosm experiments is possible and needs to ascertain the effects of ecologically relevant doses of pesticides on different endpoints.

New information about any one piece of the puzzle often opens new opportunities to test presumed relationships (e.g., Davidson 2004). As more information on the actual geographic pattern of pesticide residues becomes available, it will be possible to test the association between pesticide uses, predominate winds, and residues that are assumed in the Davidson study. Similarly, information on pesticide residues would allow a direct test of the relationship between the geographic patterns of residues and the geographic patterns of amphibian population declines.

V. ASSESSING CAUSALITY

For complex large-scale phenomena that cannot be experimentally manipulated, causality is not proven by a single study but rather inferred from the weight of evidence from multiple studies, both observational and experimental. Epidemiologists have long struggled with how to infer causality from observational studies and from multiple strands of evidence (Susser 1986; Fox 1991). Furthermore, epidemiologists regularly deal with the problem of assessing the causes of events with multiple factors, operating on both large temporal and spatial scales. To assess causality, epidemiologists have developed multiple criteria. These same techniques may be useful in evaluating the causes of amphibian declines.

Seven criteria are commonly used in assessing causality. The simplest criterion is *time order*. Does a supposed cause precede the effect? This criterion is useful for rejecting hypothesized causes that occur after the effect. Because there is an infinite number of events that can precede an effect, meeting this criterion cannot confer support for a hypothesized cause. In some cases where a supposed cause precedes an event by a long time, *time order*

can argue against the cause, unless there is a plausible mechanism for the time-lag. This may be the case for organochlorine pesticides that have been in heavy use since the 1950s (Aspelin 2003). Their use long preceded amphibian declines and, therefore, may not explain recent declines. A number of authors (Stebbins and Cohen 1995; Sparling *et al.* 2001; Davidson 2004) have suggested that organophosphate and carbamate pesticides may be contributing to declines, in part because the pesticides came into widespread use in the late 1960s and amphibian population declines, at least in western United States, are believed to have started in the early 1970s.

A second criterion for assessing causality is *biological plausibility*. Is the hypothesis consistent with known biological processes and other scientific facts? For the pesticide hypothesis, probably the most difficult question in this regard is how extremely low levels of pesticides can either cause or contribute to mass mortalities. Work by Hayes (e.g., Hayes *et al.* 2002) indicates that levels as low as parts per billion may have dramatic effects on amphibian development. Currently the most plausible mechanism whereby low-level exposures could lead to dramatic population declines is that exposures to pesticides impair immune function, leading to mass die-offs from disease or pathogens present in the environment.

A *probability criterion* is used to determine if the observed associations between a supposed cause and effect are statistically significant. With the *strength of association criterion* one evaluates how strongly associated a supposed cause is with its effect. *Strength of association* means looking beyond simple statistical significance, and examining the magnitude of effect or its relative risk compared to other explanatory factors. Because causality is ultimately assessed across multiple studies with widely different approaches, the *consistency upon replication criterion* appraises whether evidence for a supposed cause is consistent over multiple studies representing different approaches, locations, or species.

The *dose-response relationship* criterion evaluates whether the incidence of effect increases monotonically with an increase in the dose of a supposed cause. A finding of dose-response relationship is strongly supportive of a causal relationship. Not all causal relationships, however, have a monotonic dose-response relationship; therefore, lack of a dose-response relationship is not strong evidence against causality. Comparisons of an effect at two levels of a supposed cause (e.g., population declines at sites with low versus high pesticide exposures) are less convincing of causality than are comparisons across multiple levels of the supposed cause, in which the effect increases with increased levels of the cause. The final criterion is *specificity*. Does only the supposed cause lead to the effect or are there other factors that also lead to that effect? Unfortunately, in amphibians there are multiple causes of decline, so none of the hypothesized causes for it score high when using these criteria.

Ultimately, only by considering multiple studies and applying criteria for causality, such as those described above, will it be possible to begin assessing the role of pesticides in amphibian declines. In this respect, the pesticide hypothesis is no different from other hypothesis for amphibian declines. The criteria described above do not lead to a simple or clear-cut determination of causality. Many of the criteria involve difficult subjective judgments, and there are no rules for how to weight the different criteria (Fox 1991). Nonetheless, these criteria provide a start in organizing a "weight-of-the-evidence" evaluation of an hypothesized cause of an event. These criteria could aid in making conscious and explicit evaluations of the weight of evidence and lead to better assessment of causality of complex phenomenon such as amphibian population declines.

VI. CONCLUSIONS

Following the news of worldwide amphibian declines, researchers in amphibian ecotoxicology expected to find links between contaminants and declines. The physiological properties and life history characteristics of amphibians would seemingly make them sensitive to environmental contamination and, accordingly, good bioindicators of the quality of aquatic and terrestrial habitats. After a decade or more of research, however, amphibians do not

appear to be any more sensitive to contaminants than are other vertebrate species. It is, therefore, unlikely that pesticides alone will explain worldwide amphibian declines. Studies examining multiple stressors are the most promising avenue of research, and may offer insight into how pesticides influence species diversity in the natural world. It seems logical that the more stressors, natural or anthropogenic, to which an organism is exposed, the greater will be the probability that it will not be able to reach the critical bar allowing completion of metamorphosis (Boone *et al.*, in press), survival over winter or successful reproduction.

Are there characteristics that may make certain amphibian populations more sensitive to the presence of a contaminant (or other) stressor? Among the 27 North American species that Semlitsch (2003) listed as either endangered, threatened, or proposed endangered/threatened, caudates and anurans were equally represented, although reports of declines in North America are most strongly associated with declines of ranids in the west. Given that there are approximately 1.7 times more North American caudates, one would expect that there should be more caudates experiencing declines all things being equal, which suggests anurans in North America may be more susceptible. Among North American anurans, Crump (2003) reported that ranids are experiencing declines at greater than the expected rates for any of the anuran genera; among tropical anurans, bufonids appear to be experiencing more than expected declines. Comparisons of characteristics of declining amphibians worldwide (as in Crump [2003] for anurans in the tropics) may help anticipate which characteristics make amphibians most susceptible to the probability of experiencing declines, and may also help implicate the cause. For instance, Crump (2003) found that species have a higher probability of declining if they have restricted geographic distributions, occupy running water as eggs and larve, or produce small clutchs. Data such as these suggest that species that are geographically isolated or with few populations should be closely monitored, with particular attention to species with certain life history characteristics (like small clutch size), and also suggests that the problem could be associated with running water which then provides a direction for experimental research and a tangible basis for analysis (e.g., of contaminants and/or pathogens). Conversely, comparisons between areas where declines have occurred (e.g., western United States) and where they have not (e.g., eastern United States), may also help understand the factors at play in declines or which buffer populations from negative effects. In multi-species studies involving a pesticide, species with relatively short larval periods appeared to be most strongly affected by chemical exposure and/or resulting changes in the food web. In species such as toads and treefrogs, however, many of the effects were apparently "positive" due to changes in the food web (Boone and Semlitsch 2001, 2002; Boone *et al.* 2004). Species with short life spans may also be more likely to experience declines if contaminant exposure (in combination with other factors) reduces juvenile recruitment into the population, especially if metapopulation dynamics are disrupted by habitat destruction. Furthermore, populations at the edge of their range may be more likely be approaching their physiological limits; the addition of a stressor may increase the probability of extinction.

It is difficult to say which pesticides pose the greatest risk to any organism, particularly amphibians, given that they are not routinely used in standard toxicological testing. The United States Environmental Protection Agency (USEPA), however, does collect information on the amount of registered pesticides that are sold in the United States and World markets. From this information, it is known that throughout the world, herbicides are the most commonly applied pesticide, followed by insecticides, and fungicides (Kiely *et al.* 2004). So, based on sheer volume of pesticides applied, herbicides could pose the greatest threat. Herbicides typically have modes of action that are unlikely to affect animals directly, although the herbicide atrazine has been found to disrupt the endocrine system (Hayes *et al.* 2002, 2003), the carrier of the herbicide glyphosate has been found to have toxic effects on amphibians (Howe *et al.* 2004), and herbicides can reduce the food base of the community (Diana *et al.* 2000; Boone and James 2003). Herbicides may, therefore, still have important negative effects on amphibian populations. Insecticides have modes of action that affect amphibians

and their food web; so while a lesser amount of active ingredient is applied, the effect could be more (or equally) detrimental. Evaluating the effects of commonly used pesticides that have widespread use may be one way to begin evaluating the effects of pesticides on amphibian populations.

The big question regarding amphibian declines is why they seemingly began at roughly the same time across the world. Such a pattern does suggest a global phenomenon and a cause that has global distribution. Pesticides and environmental contamination could have served as a trigger, especially if they do make amphibians more susceptible to stressors already present in the environment (e.g., pathogens or disease). Evaluation of critical interactions in experimental manipulations and examining other factors that may operate in the same manner as pesticides might lead to better understanding of the causes of decline and enable formulation of better management policies for protection of amphibian populations.

VII. ACKNOWLEDGEMENTS

David Bradford introduced us to the relevant epidemiological literature and has long advocated using epidemiological criteria to evaluate causes of amphibian decline.

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