CARBARYL EFFECTS ON AMPHIBIAN SPECIES WITH IMPLICATIONS FOR FURTHER RESEARCH

A Literature Review
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Abstract. Declines in amphibian populations have been reported worldwide. Increased pesticide use is hypothesized to contribute to the loss of amphibian species. A comprehensive review of literature discusses water contamination by the insecticide, carbaryl, to illustrate the challenges of ecotoxicology and the study of amphibian declines. Because amphibian survival is determined by complex interactions of multiple abiotic and biotic factors, it is logical to conclude that better understanding of the declines will be found through the examination of the interactive effects of multiple stressors. In addition to long-term research and monitoring, further study of multiple stressors could provide data necessary to slow the rate of amphibian declines and may serve as a model for addressing the global issue of decreasing biodiversity.

Introduction

Brief History of Amphibian Declines

Amphibian populations have been declining worldwide since the 1970s in a trend believed to be too dramatic and extensive to be attributed to natural demographic variations (Stuart et al. 2004). The estimated rate of extinction currently surpasses fluctuations recognized over the last 100,000 years (Eldridge 1998). Thirty-four species of amphibians are reported to have vanished between the years 1500 and 2004. Nine of those extinctions occurred between 1980 and 2004, and an additional 113 species have apparently disappeared since 1980, but have not been formally classified as “extinct” (Stuart et al. 2004).

The loss of amphibian populations became a global concern in 1989, at the First World Congress of Herpetology (Collins and Storfer 2003). In 1990, a U.S. National Research Council Workshop began to investigate the phenomenon (Wake 1991). By 1993 over 500 different populations of frogs and salamanders were recognized as declining or listed as species of special conservation concern (Alford and Richards 1999). At the Third World Congress of Herpetology in 1997, the critical need for further research to address the increasing threat of potential amphibian extinction was identified (Collins and Storfer, 2003).
In 2004, the Global Amphibian Assessment (GAA) of the World Conservation Union (IUCN), reported that approximately 32.5% of amphibian species were globally threatened, 7.4% were critically endangered, and 22.5% were not assessed due to lack of data. At least 43.2% of amphibian species were experiencing population decrease, while 0.5% was increasing, 27.2% were stable, and 29.1% have an unknown trend (Stuart et al. 2004).

**Significance of Amphibian Declines**

Loss of biodiversity is a global environmental concern (Wake 1991, Blaustein et al. 1994a). It is important to understand amphibian declines because it could serve as a model for addressing the general issue of decreasing global biodiversity (Houlahan et al. 2000, Alford et al. 2001). Amphibians are considered biomarkers of environmental stress (Blaustein et al. 1994, Blaustein and Wake 1995), and the severe decline in amphibian biodiversity could be an indication of serious environmental problems often associated with human activities (Wake 1991). Amphibian losses are occurring simultaneously, expansively, and rapidly. Most notably, population declines are occurring in protected natural areas, so efforts to protect species have not been effective (Collins and Storfer 2003).

Amphibians provide ample biomass and biodiversity to their environment (Blaustein et al. 1994a, Boyer and Grue 1995). The species have important roles in the food web. Amphibians are predators, as well as, prey for other trophic levels (Blaustein et al. 1994a, Wake 1991) and they may be herbivorous, carnivorous, or omnivorous, depending on species and life stage (Zug 1993). The ecological impact of loss of
amphibian biodiversity is difficult to forecast; but, because of their role in trophic
dynamics, the worldwide reduction of amphibian species and subsequent alteration of the
biological community could have significant consequences for other organisms
(Blaustein et al. 1994a). The importance of amphibian populations may not be fully
recognized until they have vanished, and the consequences could be immense and
irreversible.

**Major Hypotheses**

Six major hypotheses to explain the cause of the amphibian population declines are introduced species, over-exploitation, land alteration, global change, disease, and the increased use of pesticides and other toxic chemicals. Studies investigating amphibian population declines demonstrate that the underlying mechanisms are complex and involve interactions among abiotic and biotic components (Blaustein and Kiesecker 2002). Many researchers agree that, generally, amphibian declines are not likely to be the result of one single factor, but rather, the product of synergistic interactions of multiple stressors (Alford and Richards 1999, Blaustein and Kiesecker 2002). Amphibian reaction to one stressor can be different when affected by the presence of another stressor (Kiesecker et al. 2001a). Therefore, studying how these factors interact should provide better understanding of the reasons for increasing rates of amphibian loss (Blaustein and Kiesecker 2002). It is especially important to examine human impacts on amphibian declines so that measures can be taken to slow the rate of extinctions (Collins and Storfer 2003).
**Introduced Species**

The introduction of non-native species can have negative consequences for amphibian populations. For example, mass introductions of predatory fish have caused an increase in amphibian exposure to predators which they have not had previous interactions with and therefore have not evolved anti-predator mechanisms against (Alford and Richards 1999). Amphibian response mechanisms that would protect them against natural predators could thereby be ineffective against the alien species (Gamradt and Kats 1996). When predatory fish are introduced to normally fish-free waters, rapid extinction of amphibian assemblages can occur (Fisher and Shaffer 1996). Predation, a direct effect, as well as indirect effects including competition, hybridization, and the introduction of pathogens, cause devastation to native amphibian populations as a result of the introduction of alien species to a habitat (Collins and Storfer 2003).

**Over-exploitation**

Ranid species are at the highest risk of over-exploitation due to extensive harvesting for frog legs for human consumption (Stuart et al. 2004). Over-exploitation has caused rapid decline in at least 50 amphibian species and is considered a major source of amphibian declines in East and Southeast Asia (Stuart et al. 2004). In the past, the frog leg trade has accounted for annual exports of approximately two hundred million frogs in Asia (until 1995) and seventy million frogs in India (by 1990) (Oza 1990). Lannoo et al. (1994) reported that harvesting for frog legs accounted for at least one third of the decline in amphibian species in one Iowa County where estimates of frog abundance decreased from at least 20 million frogs in 1920 to about 50,000 in 1992.
Land-use/land-cover change

Alford and Richards (1999) asserted that “habitat modification is the best documented cause of amphibian population declines.” Meyer and Turner (1992) divided this hypothesis into two sub-categories: land-use change and land-cover change. A change in the way people use the land is termed land-use change, while land-cover change is the alteration of the “physical or biotic character of the land surface,” (Meyer and Turner 1992). Land-use change is basically disturbance of the environment; for example, the introduction of non-native species or pollution of the water, which has various adverse effects on amphibian species (see Increased Use of Pesticides and Other Chemicals below). Land-cover change typically involves habitat loss or food scarcity; for example, wetland drainage, deforestation, and agricultural intensification. Almost 120,000 ha of wetlands are lost each year (Boyer and Grue 1995). Petranka (1998) declared that the biggest threat facing the smallmouth salamander, *Ambystoma texanum*, is the conversion of such bottomland habitats to agricultural areas. High-traffic roadways, which may have considerable impacts on anuran populations, are another example of land-cover change (Lamboureux and Madison 1999). Land-use change and land-cover change are noted to cause of global loss of biodiversity, including the loss of amphibian species (Collins and Storfer 2003).

Global Change

The global change hypothesis encompasses various changes in the planet. Global climate change as a result of depletion of stratospheric ozone is one concern. For
example, certain species of amphibians require fishless ponds for breeding and thus depend on ephemeral pools that cannot sustain populations of fish. Altered precipitation patterns, as observed in the neotropics, can cause ponds to fill later and dry earlier, resulting in increased density in the limited breeding sites (Donnelly and Crump 1998). Density mediated effects of increased competition and predation may then induce shifts in amphibian distributions (Donnelly and Crump 1998).

Stratospheric ozone depletion is also a source of increased ultraviolet-B (UV-B) radiation (290 – 320 nm) at the terrestrial surface (McKenzie et al. 2007), which could potentially affect amphibian populations in certain locations. Blaustein et al. (1994b) measured levels of photolyase, a photoreactivating DNA repair enzyme that mends UV-B damage, in eggs and oocytes of ten amphibian species (\textit{Xenopus laevis}, \textit{Rana cascadae}, \textit{Hyla regilla}, \textit{Bufo boreas}, \textit{Taricha granulosa}, \textit{Ambystoma gracile}, \textit{Ambystoma macrodactylum}, \textit{Plethodon dunni}, \textit{Plethodon vehiculum}, and \textit{Rhyacotriton variegatus}), and performed field experiments to measure the effects of UV-B on hatching success of three anuran species (\textit{Rana cascadae}, \textit{Bufo boreas}, and \textit{Hyla regilla}). The study found the variation in levels of photolyase among species to be correlated with exposure to sunlight of natural egg laying locations. Furthermore, Blaustein et al. (1994b) found a remarkable correlation between the population status of the species tested for hatching success, and their ability to repair UV-B damage: the species experiencing drastic population declines (i.e. \textit{Rana cascadae} and \textit{Bufo boreas}) were those with the lowest photolyase activity.

Note that, with the exception of laboratory-reared \textit{Xenopus laevis}, all amphibian subjects were collected from mountainous populations, which may be more at risk for
exposure to high levels of UV-B irradiance than populations at lower elevations. Kerr and McElroy (1993) reported increases in UV-B radiation (35% per year in winter, and 7% per year, in summer for 4 years) associated with detections of decreasing ozone at the same latitudes (44ºN) in Canada as the field sites used by Blaustein et al. (1994b). Most importantly, Blaustein et al. (1994b) related adverse effects of UV-B in spring field trials to the observation by Kerr and McElroy (1993) that rising levels of UV-B in late spring may have a much greater impact on some species if they occur at critical stages of development.

Embryos may be at risk for lethal and sublethal effects resulting from exposure to UV-B radiation. Amphibian larvae and adults are generally able to seek refuge from ambient UV-B, while embryos may be exposed to continuous levels of sunlight because they are stationary and must endure the environmental conditions in the location in which they are deposited (Zaga et al. 1998). Anzalone et al. (1998) observed high rates of embryonic mortality in *Hyla cadaverina* and *Taricha torosa* from exposure to solar UV-B radiation at natural breeding sites in California (34ºN latitude, 290 m altitude), while protection from solar UV-B resulted in significantly greater hatching success. Amphibian eggs that are exposed to increased ambient levels of UV-B radiation have decreased hatching success and suffer sublethal effects that alter growth and development, behavior, anatomy, and physiology (Blaustein et al. 2001b). Constant mortality in early amphibian life stages may eventually lead to declining population (Blaustein and Kiesecker 2002).

**Disease**
Emerging Infectious Diseases (EIDs) are diseases that have recently increased in incidence or geographical range, have impacted new host populations or increased in pathogenicity, and developed from newly discovered or newly evolved pathogens (Daszak et al. 2003). Three pathogens inducing EIDs that are believed to contribute to amphibian declines are *Batrachochytrium dendrobatidis, Saprolegnia ferax,* and *Ambystoma tigrinum* virus (ATV) (Blaustein and Kiesecker 2002).

*Batrachochytrium dendrobatidis* is a fungus responsible for the disease, Chytridiomycosis, which has been linked to mass die-offs in numerous amphibian species around the world, especially in Latin America (Lips et al. 2006). Increasing evidence suggests that *Batrachochytrium dendrobatidis* is a recently evolved pathogen contributing to amphibian declines (Lips et al. 2006, Daszak et al. 2003, Morehouse et al. 2003, Weldon et al. 2004). Furthermore, studies suggest that amphibian susceptibility to the disease may be influenced by global climate change (Berger et al. 2004, Pounds et al. 2006, Bosch et al. 2007).

*Saprolegnia ferax,* a pathogenic oomycete, has contributed to significant embryonic mortality and has been proposed to be a contributing factor in the declines of the boreal toad (*Bufo boreas*) and the Cascades frog (*Rana cascadae*) in the Pacific Northwest United States (Daszak et al. 2003). Kiesecker et al. (2001a) showed that UV-B radiation has a synergistic effect on boreal toads in the presence of the pathogen. Increased mortality was observed in *Saprolegnia*-infected toads exposed to increased levels of UV-B radiation (Kiesecker et al. 2001a). Other factors may influence amphibian susceptibility to the pathogen as well. Kiesecker et al. (2001b), for instance, found that introduced fishes are a primary source of *Saprolegnia* infection. The disease
may be directly transmitted to amphibian embryos exposed to infected fish or indirectly transmitted by soil substrate carrying the pathogen (Kiesecker et al. 2001b).

The iridovirus, *Ambystoma tigrinum* virus (ATV) can also be transmitted directly or indirectly by salamanders exposed to ATV (Brunner et al. 2007). Cofactors may also play a role in the spread of the ATV (Blaustein and Kiesecker, 2002). For example, Rojas *et al.* (2005) observed that temperature affected percent mortality and time to death of salamanders exposed to ATV. When exposed at 26º C, most of the salamanders survived, but at 18º C all of the salamanders died, and at 10º C almost all of the salamanders died as well (Rojas et al. 2005). The effects observed in this study could provide reason for cyclic viral epizootics of *A. tigrinum* in nature which could be influenced by seasonal variations in water temperatures (Rojas et al. 2005). Exposure to the widely used herbicide, atrazine may also influence salamander susceptibility to ATV and mortality and contribute to ATV epizootics (Forson and Storfer 2006a, b)

While pathogens may be present in healthy animals, a weakened immune system allows the disease to take effect (Alford and Richards 1999). Therefore, the sublethal effects of other environmental stressors could make amphibians more susceptible to disease (Carey 1993, Kiesecker 2002). Of particular interest to researchers are the impacts of global change on amphibian susceptibility to and mortality by disease (Blaustein and Kiesecker 2002).

**Increased Use of Pesticides and Other Chemicals**

Pollutants are able to stress amphibians in a variety of ways (Blaustein and Kiesecker 2002). They can cause mortality, change behavior, decrease growth rates,
disrupt endocrine functions, and suppress the immune system (Alford and Richards 1999). Studies have revealed that amphibian species are as sensitive, if not more sensitive, than other aquatic organisms when exposed to certain contaminants (Boyer and Grue 1995, Blaustein and Wake 1995). This is likely because they are susceptible to dermal absorption of toxicants in the water at all life stages (Boyer and Grue 1995). Amphibians respire through a moist, permeable skin and lay unshelled eggs that are directly exposed to soil, water, and sunlight, and easily absorb environmental contaminants (Blaustein and Kiesecker 2002, Blaustein et al. 2003). Considerable disturbance of the aquatic ecosystem can result from chemical contamination (Relyea and Hoverman 2006). Owing to the physiological traits and central position in the food chain, the effects of contaminants on amphibians are expected to be severe (Broomhall 2005).

According to Ongley (1996), agriculture is the number one supplier of water pollution worldwide. Chemical fertilizers and pesticides are the leading source of water contamination by agriculture (Horne 2001). The use of chemicals in agriculture has increased drastically since the late 1940s (Boyer and Grue 1995), and with the biotechnological development of crops resistant to pesticide applications, the use of agrichemicals is growing rapidly. In the first eight years that genetically modified crops were available to farmers, the use of pesticide resistant corn, soybean and cotton crops led to a 122 million pound increase in pesticide use (Benbrook 2004).

Pesticides enter aquatic systems through direct application, terrestrial runoff, erosion, and aerial drift (LeNoir et al. 1999, Relyea and Hoverman 2006). Pesticides may even affect amphibians in remote and considerably undisturbed habitats through
atmospheric transport (Blaustein et al. 2003). Many amphibian species inhabit very small ephemeral pools where even minute amounts of pesticide can result in significant chemical concentrations (Sanders 1970, Blaustein et al. 2003). In addition, breeding and larval development are periods when amphibian species are generally restricted to their aquatic environment. These critical periods in amphibian life histories occur during the warmer seasons of the year, typically during growing seasons when agrichemicals are applied to the land. Because species remain in their aquatic habitat for months or even years, they may be exposed to contaminants numerous times (Boone et al. 2001) due to repeated applications of agrichemicals. For instance, carbaryl is recommended by one manufacturer (Ortho) to be reapplied every 7 to 10 days, or as needed (Boone et al. 2001). Thus, amphibian species may be quite vulnerable to agricultural contaminants (Blaustein and Kiesecker 2002).

**Lack of Protection against Water Contamination**

The need for increased protection of amphibians and other aquatic species through the development of water quality criteria has been recognized by researchers (Boyer and Grue 1995). Though the number of ecotoxicology studies published annually has increased tremendously since 1992 (Relyea and Hovermann 2006), there is little data to establish how contaminants affect amphibian populations in the environment (Blaustein and Kiesecker 2003).

**Limitations in Testing**
There are thousands of different pesticides used around the world (Relyea and Hovermann 2006), not to mention countless other contaminants that enter aquatic systems (Koplin et al. 2002). As a requirement under the Toxic Substances Control Act, toxicity tests must be conducted to determine the hazardous effects of various chemicals (Relyea and Mills 2001). Due to financial and time limitations, however, acute toxicity analyses employing only a few model organisms are often used to determine acceptable levels of a chemical concentration that is safe for humans and non-target organisms (Cooney, 1995).

LC50 tests are the most commonly utilized of these assessments (Relyea and Mills 2000). An LC50 value is an estimate of the lethal concentration of a chemical necessary to kill 50% of a test population. The estimate is used to determine the maximum amount of a toxicant that can be tolerated by non-target species and to set environmental safety standards; however, because of short exposure periods (and generally high chemical concentrations) used these values may be overestimated (Relyea and Mills 2000), (see LC50 analyses below).

Furthermore, acute toxicity tests may detect the toxicity that directly affects a species, but the results of these experiments do not accurately reflect the complex effects of chemical contamination in an actual aquatic community (e.g., sublethal effects, indirect effects, additive effects) (Blaustein and Kiesecker 2002). Various biotic (e.g., predators, competitors, food resources) and abiotic (e.g., temperature, pH, UV-radiation) factors that are present in the environment can interact with the amphibian, with the chemical, or with other factors to produce effects that cannot be predicted in single-factor studies (Saura-Mas et al. 2002).
Moreover, pesticide registration does not require amphibian tests, thus experiments with fish and aquatic invertebrates have generally determined the tolerance level for aquatic chemical exposure of amphibian species (Bridges et al. 2002). Because fish and other aquatic invertebrates have different sensitivities to toxicants than amphibians, the evaluation of aquatic chemical exposure to amphibians is of extreme importance in regulating agrichemicals (Bridges et al. 2002). Amphibian species are frequently, but not always, more sensitive to water contamination than other vertebrate species, and sensitivities can differ depending on the contaminant being tested (Bridges et al. 2002). Also, sensitivity to toxicants can vary among species (Bridges and Semlitsch 2000), among populations of a single species (Bridges and Semlitsch 2000) and among life stages (Widder and Bidwell 2006). In addition, the sensitivities of various species must not be determined solely by the results of acute toxicity tests, but with additional studies including chronic exposure and sublethal concentrations to gain more accurate results on species’ sensitivities (Bridges et al. 2002).

**Limitations in Interpretations**

Pesticide concentrations detected in aquatic systems are frequently lower than those used in toxicity experiments. However, environmental concentrations are generally considered “snapshots in time” rather than detections of maximum concentrations (i.e. at the time of application) in an aquatic system (Relyea and Hovermann 2006). The accumulation and the rate of breakdown of a chemical within a body of water can be affected by various factors including the size of the water body, timing of application, pH, temperature, sunlight exposure and other factors. Koplin et al. (2002) states that
astonishingly little is certain about the degree of environmental occurrence, transport, and ultimate fate of many synthetic organic chemicals after their intended use. Lack of extensive data on pesticide concentrations in the environment thus restrict the conclusions that can be drawn from toxicity studies (i.e. the relevance of the concentrations used may not be clear) (Relyea and Hovermann 2006).

Agrichemicals (and other contaminants) that are not intended for or legally registered for application to aquatic systems still appear in these environments (e.g. Thompson et al. 2004, Koplin et al. 2002) as a consequence of runoff, areal drift, erosion, direct overspray, etc. (Peterson et al. 1994). Until we know how these agrichemicals are affecting amphibian species, we cannot confirm that they are not having serious adverse effects on amphibian species and the ecosystem. Hence, further research is needed to determine chemical concentrations of pesticides in aquatic systems and the effects of these chemicals on non-target organisms to better understand the threat the contaminants pose to amphibian species (Relyea and Hovermann 2006).

**Amphibian Declines near Areas of Intense Cultivation**

A relationship exists between amphibian declines and proximity to agricultural lands. Pesticides are carried downwind and deposited in amphibian habitats. For example, vast quantities of agrichemicals are applied to the San Joaquin Valley of California each year (Sparling et al. 2001). The valley is one of the most highly cultivated and productive regions in the world, and relies on the use of millions of kilograms of pesticides, particularly organophosphate (OP) pesticides (Aston and Seiber 1997). Through volatization and airborne transport by dominant wind patterns, the
pollution is carried from the San Joaquin Valley and eventually deposited in the nearby
Sierra Nevada mountains (Zabik and Seiber 1993, Aston and Seiber 1997, Le Noir et al.
1999). Zabik and Seiber (1993) showed that considerable amounts of OP insecticides (as
high as 100 ng/L for diazinon, 10 ng/L for chlorpyrifos, and 10 ng/L for paration) used in
the San Joaquin Valley are transported to the Sierra (e.g., Sequoia National Park).
Between 50% and 100% of the precipitation and water samples taken from different sites
(i.e. at 533 m elevation and at 1,290 m elevation in Sequoia National Park and at 2,200 m
elevation in the Lake Tahoe Basin) within the Sierra Nevada mountains, contained OPs
and other currently used pesticides (as high as 85 ng/L chlorothalonil, 13 ng/L
chlorpyrifos, 24 ng/L malathion, 19 ng/L diazinon, and 2 ng/L trifluralin,
hexachlorocyclohexane, and endosulfan) (McConnell et al. 1998). An estimated annual
loading of chlorpyrifos, an organophosphate, was determined to be 24 to 31 kg/year for
the Sequoia National Park area (McConnel et al. 1998). Concentrations in air and water
correlated roughly with peak pesticide application periods in the Valley (McConnel et al.
1998). The detected concentrations are less than acute toxicity values of amphibian
species, though additive, synergistic, and indirect effects of the pesticides must be
considered (LeNoir et al. 1999).

The most severe declines in amphibian species in California have been found in
the Sierra Nevada Mountains (Drost and Fellers 1996, Fellers and Drost 1993). Air
masses laden with ozone, aerosols, and agrichemicals are moved into the Sierra causing
deteriorated air quality in the foothills and the mountains (Aston and Seiber 1997).
Widespread ozone damage has occurred throughout the Sierra (i.e., possible increased
UV radiation effects) and there is concern that agrichemical effects may be in part
responsible for declining number of native amphibians (Boyer and Grue 1995). In some areas there have been reports that no frogs can be found, though only 10 yr earlier there were well documented thriving populations (Aston and Seiber 1997).

Sparling et al. (2001) measured cholinesterase activity and residues in a California anuran species, *Hyla regilla*, and found that the frequency of detections of pesticide residues, as well as concentrations of pesticides in amphibian tissues, correlated with patterns of consistent agrichemical drift and areas of amphibian declines. Cholinesterase-inhibiting pesticide exposure could therefore be responsible for sharp population declines in frog species in Sierra Nevada range in California (Sparling 2001, Davidson 2004).

**Carbaryl**

**Background**

Subsequent to the prohibition of organochlorine (OC) pesticides in the 1970s, the use of organophosphate (OP) and carbamate pesticides increased dramatically (Sparling et al. 2000). The increase in OP and carbamate pesticide use immediately preceded or coincided with amphibian declines, which are believed to have begun in the early to mid-1970s in the Western United States (Jennings 1996). Carbaryl (1-naphthyl-N-methylcarbamate) is a broad-spectrum insecticide, better known by the trade name SEVIN, that is common in agriculture, forestry, lawn and garden use throughout the United States and Canada (Rohr et al. 2003). It is a neurotoxin that kills amphibian species by inhibiting acetylcholinesterase (Relyea 2005) which results in the accumulation of acetlycholine in the synapse, causing the nerve to repeatedly fire (Zaga
et al. 1998). Examining the effects of carbaryl on amphibian species is important because cholinesterase-inhibiting pesticides are most strongly associated with amphibian declines and carbaryl may serve as a model chemical for understanding the effects of carbamate and organophosphates (i.e., cholinesterase inhibitors) (Cox 1993, Rand 1995).

**Concentration and Breakdown in the Environment**

Carbaryl is one of the most frequently detected pesticides in urban streams (Phillips et al. 2007, Schreder and Dickey 2005). Environment Canada calculated the Expected Environmental Concentration (EEC), the expected concentration resulting from maximum proposed application rate to a 15 cm deep water body, to be 3.667 mg/L (Peterson et al. 1994). Past studies have found the insecticide at concentrations as high as 4.8 mg/L (Norris et al. 1983), although detections of concentrations greater than 0.01 mg/L have not been reported recently (Brena et al. 2005, Koplin et al. 2002, Phillips et al. 2007).

It is possible that greater concentrations are present in the environment at levels that may have adverse effects on amphibian species (see Limitations in Interpretations above). For instance, levels of the insecticide have been determined in larger water bodies where the significant levels of carbaryl are less likely to exist when compared to shallow waters where smaller amounts of the toxicant result in higher concentrations. Also, carbaryl decomposes rather quickly under most environmental conditions (Sparling et al. 2001); therefore peak concentrations of the contaminant have not necessarily been detected (Relyea and Hovermann 2006). Studies on the concentration of carbaryl in
small ephemeral pools receiving direct overspray could provide a “worst-case scenario” for the insecticide concentrations in the environment.

Although recent detections of carbaryl in the environment are at low concentrations, frequent detections of high levels of 1-naphthol, the major breakdown product of carbaryl, in groundwater and surface water have been observed (Brena et al. 2005). A study conducted by Brena et al. (2005), for example, did not detect significant levels of carbaryl, but considerable amounts of 1-naphthol were detected more often than any other contaminant. Brena et al. (2005) suggests that the breakdown of carbaryl occurred prior to sampling, probably catalyzed by enzymes from soil microorganisms; though the detection of 1-naphthol may serve as an indicator that short-lived agrochemicals also reach the groundwater. 1-Naphthol could be of concern because it is more toxic than carbaryl to certain species, including freshwater fish (Tilak et al. 1981, Brena et al. 2005).

Carbaryl is broken down by various pathways in the environment. Hydrolysis by bacteria and aqueous photolysis are the most important processes for breakdown in water (Brena et al. 2005). Carbaryl has a half-life of about four days under most environmental conditions (Miller 1993). Other environmental factors can affect the rate of carbaryl breakdown in the environment (e.g., increased sunlight and UV-B radiation, pH, temperature, natural organic matter, nitrate) (Miller and Chin 2002), therefore the duration of carbaryl in the environment is highly dependent upon environmental conditions (Relyea 2003). For example, the pH of natural wetlands is generally 5.0-9.0; at pH 9.0, the half-life of carbaryl is approximately 0.1 day, whereas it could take almost
4 yrs for carbaryl to break down at pH 6.0 (Relyea 2003). The duration of exposure to carbaryl is thus highly variable (Relyea 2003).

**Direct Effects**

**Time-to-Death Assays**

Time-to-death assays are toxicological tests that examine mortality at a single concentration across time; thus measuring the rate of mortality as a result of chemical exposure (Bridges and Semlitsch 2000). Bridges and Semlitsch (2000) measured time-to-death in tadpoles under laboratory conditions to examine carbaryl sensitivities among nine ranid species, within a single species (i.e. among populations), and within populations (i.e. among families) of a single species.

Significant differences in time to death among the nine ranid species were observed. *Rana aurora* was the most tolerant species followed by *R. pretiosa, R. palustris, R. sphenoecephala, R. bairi, R. clamitans, R. boylii, R. areolata,* and *R. sylvatica* was the most sensitive to carbaryl. It is interesting to note that two of the three western U.S. species that have experienced the greatest declines, *R. pretiosa* and *R. aurora* were the most tolerant of carbaryl; while the third, *R. boylii,* had average tolerance. Bridges and Semlitsch (2000) attributed this observation to these species becoming locally adapted to harsh environmental conditions (i.e. pesticide exposure).

Time to death also varied significantly among *R. sphenoecephala* populations indicating significant variation in carbaryl tolerance within populations throughout the species’ range (Bridges and Semlitsch 2002). The differences in sensitivity were
suggested to be a result of “lingering effects of a past environmental selective pressure of unknown intensity,” (Bridges and Semlitsch 2002).

Semlitsch et al. (2000) conducted time-to-death assays on *Hyla versicolor* tadpoles under laboratory conditions to measure carbaryl tolerance of tadpoles from different families and correlated data with mean performance of each family reared in field enclosures (i.e. enclosed natural ponds). Significant variation in carbaryl tolerance was observed among tadpoles within a single population of the gray treefrog, *Hyla versicolor*. Semlitsch et al. (2000) believed that the intrapopulation differences in carbaryl tolerance, although affected by genetic variation in body size, more likely developed from environmental maternal contributions (e.g., egg size and yolk quality), which could be important in amphibian population dynamics. Further, data indicated that genotypes tolerant to chemical contamination may be less fit in other ways as a result of natural selection. This study demonstrated that understanding environmental and genetic variation in chemical tolerance is critical to identifying species vulnerability to contaminants and thus understanding species declines (Semlitsch et al. 2000).

**LC50 Toxicity Assays**

Relyea and Mills (2001) demonstrated that results of LC50 tests may be significantly overestimated. For example, Bridges (1997) estimated that the concentration necessary to kill 50% of the population in two days (LC50$_{2-d}$) for *Hyla versicolor* to be 12.9 mg/L. The LC50$_{4-d}$ for *Hyla versicolor*, determined by Zaga et al. (1998), was 2.5 mg/L, which is consistent with LC50 estimates for other larval anurans (Marian et al. 1983; Marchal-Segault 1976). However, Relyea and Mills (2001) found
that carbaryl concentrations of only 0.05 mg/L and 0.09 mg/L caused up to 97% mortality of *H. versicolor* tadpoles over 10 d (i.e. half of the population was exterminated in about 7 d). Therefore, lower concentrations of carbaryl may cause mass mortality of amphibians, only over a longer period of time. Thus, LC50 tests should be conducted using low and environmentally relevant concentrations, and testing periods should be extended by at least a few days, to obtain more exact estimates of acute toxicity (Relyea and Mills 2001).

Relyea (2003) used five concentrations of carbaryl (0.03, 0.3, 1.6, 3.2, and 6.5 mg/L) to determine LC50 estimates for six anuran species of tadpoles in the absence and presence of predator cues (see Indirect Effects below). The LC50 estimates for the direct effects of carbaryl (chronic) exposure on *Rana sylvatica*, *Rana pipiens*, *Rana clamitans*, *Rana catesbeiana*, *Bufo americanus*, and *Hyla versicolor* were 1.2 mg/L, 2.2 mg/L, 2.6 mg/L, 2.3 mg/L, 3.4 mg/L, and 2.5 mg/L, respectively. Previous LC50 values, obtained over shorter exposure durations (3-4 d), ranged from 2.5 to 18 mg/L (Marchal-Segault 1976, Marian et al. 1983, Bridges 1997, Zaga et al. 1998) whereas LC50 values ranged from 1.2 to 3.4mg/L in this study (Relyea 2003). The LC50 estimates of anurans obtained in this study may be more appropriate for natural wetlands where the pH is low and carbaryl breakdown is slower (see Concentration and Breakdown in the Environment, above) (Relyea 2003).

**Sublethal Effects**

Carbaryl is found in field concentrations (≤4.8mg/L, Norris et al. 1983) generally lower than those that are directly lethal to amphibian larvae (Boone et al. 2001).
Therefore it is important to study the impact of the pesticide at low, sublethal concentrations (Bridges, 1999). The sublethal effects of a pesticide are the effects of chemical exposure that is not toxic enough to cause direct mortality. Sublethal levels of direct carbaryl exposure have the potential to alter population dynamics because they can cause amphibian species to have a shorter larval period and increased rates of metamorphosis, associated with smaller size (Bridges 1997). Carbaryl may directly affect metamorphosis by activating corticotropin-releasing hormone, a stress hormone that initiates premature metamorphosis in tadpoles in drying and stressed conditions (Denver 1995). Earlier metamorphosis is correlated with lower overwinter survival, lower lifetime reproduction, and suppressed immune function (i.e. increased vulnerability to disease) (Smith 1987; Carey et al. 1999). Further, larval performance is highly correlated with adult health, and, thus, survival (Wilbur 1997). Therefore, low levels of carbaryl could cause a population to decline slowly over time (Bridges and Semlitsch 2000).

Sublethal effects may be more valuable in assessing sensitivity to contaminants than lethal effects (Little et al. 1990) because the sublethal effects may be more detrimental to amphibian populations than effects of direct mortality (Peacor and Werner 2001).

Rohr et al. (2003) conducted static renewal laboratory tests that exposed streamside salamander (Ambystoma barbouri) embryos to 0.5, 5, and 50 µg/L carbaryl for 37 d. The experiment was developed to represent the direct effects of very low sublethal levels of carbaryl believed to persist in aquatic ecosystems for significant periods of time (Rohr et al. 2003, Gibbs et al. 1984). Carbaryl, at 50 µg/L, produced asymmetrical larvae with missing limbs and digits, and caused significant mortality in salamander larvae; thus A. barbouri could be highly sensitive to contaminants (Rohr et al.)
Also, there was greater mortality in larvae than embryos, which Rohr et al. (2003) consider to be a result of either bioaccumulation (i.e. accumulation of chemicals over time) or greater larval susceptibility to chemical exposure (i.e., jelly coating of eggs could offer protection to embryos). Carbaryl also caused larvae to spend more time in refuge and decreased activity. Lethargic larvae were least likely to display standard responses to hunger. The sublethal effects of carbaryl exposure (e.g., deformities, lethargy, increased use of refuge) could cause less effective foraging, increased predation, suppressed immune response, and ultimately decreased survival in streamside salamanders (Rohr et al. 2003).

Bridges (1997) exposed tadpoles to sublethal levels of carbaryl to monitor the direct effects of the insecticide exposure on general activity and swimming performance. Activity and swimming performance was recorded for each individual tadpole prior to chemical exposure and at 24, 48, 72, and 96 h for the duration of exposure. Then tadpoles were placed in fresh water and monitored at 24 and 48 h post-exposure.

Carbaryl caused nearly 90% reduction in activity at 3.5 mg/L, a relevant environmental concentration. Tadpole activity decreased thirty minutes after exposure and did not change during subsequent observations; therefore the effects of carbaryl are demonstrated within a very short time after introduction into the aquatic environment (Bridges 1997). At 48 h post-exposure, tadpole activity recovered for subjects exposed to 3.5mg/L, but significant recovery was not observed at higher concentrations. Bridges (1997) concluded that acute exposure to sublethal levels of carbaryl produces a reduction in both the activity and swimming performance in *R. blairi* tadpoles. The effects
observed in this study showed that carbaryl may strongly impact critical tadpole life
history functions and indirectly affect adult fitness and survival (Bridges 1997).

Bridges and Semlitsch (2000) monitored spontaneous swimming activity of ranid
tadpoles exposed to a 2.5 mg/L. Decreased activity levels were observed in all nine ranid
species exposed to carbaryl. Activity levels also differed among eight _R. sphenophala_
populations exposed to carbaryl, indicating differential sensitivities throughout the
species range (Bridges and Semlitsch 2000). Variation was not observed among families
of _R. sphenophala_ (Bridges and Semlitsch 2000).

Saura-Mas _et al._ (2002) exposed _Hyla versicolor_ eggs, and tadpoles to 3.5 mg/L
carbaryl to test for sublethal effects on survival, mass, and time to metamorphosis. The
anurans were exposed to the chemical in the laboratory for 48 hours; then, the subjects
either remained in the laboratory or were transferred to field enclosures (e.g. natural
ponds enclosed to hold tadpoles and exclude competitors and predators).

The study found no direct effect of chemical exposure on tadpoles. However,
animals reared in the laboratory had longer larval periods, larger mass at metamorphosis,
and better survival than those reared in the field, which were likely effects of
competition, predator cues (e.g., of predator presence outside of enclosures) and
fluctuating abiotic factors (e.g., temperature, pH, dissolved oxygen) under field
conditions. Thus, the effects observed in laboratory studies may be different than the
effects of the toxicant in the field (see Field Studies and Multiple Stressors below).
Further, the dominant effects of the insecticide on amphibians in the aquatic environment
are believed to be indirect effects (Boone and Semlitsch 2001, 2002). The pesticide
exposure was not necessarily inconsequential, but may have affected endpoints not
considered in this study (Saura-Mas et al. 2002). While single-factor studies are important, they do not necessarily reveal the consequences that pesticides have in the environment (Mills and Semlitsch 2002).

**Indirect Effects**

**Laboratory Studies**

The effects of carbaryl interact with other environmental factors or alter the biological community thereby having indirect effects on a species (Mills and Semlitsch 2004). Bridges (1999) exposed *H. versicolor* tadpoles to two sublethal levels of carbaryl (1.25 and 2.50 mg/L) expected to occur in the environment post-application, to determine effects on tadpole activity and predator avoidance behavior. Bridges (1999) observed that chemical exposure caused tadpoles to reduce use of refugia in the presence predator (i.e., adult *Notophthalmus viridiscens*) chemical cues, thus increasing risk of predation. Tadpoles exposed to carbaryl also spent more time in refugia when a predator cues were not present, thereby limiting their food resources. Carbaryl exposure also caused an overall decrease in activity of tadpoles, and produced longer larval periods and smaller size at metamorphosis. The effects observed in this study could diminish adult fitness, performance, and survival (Wilbur 1997, Smith 1987).

Boone and Bridges (1999) tested the effects of temperature on carbaryl potency using *Rana clamitans* tadpoles. Tadpole survival was negatively correlated with the interaction of temperature, chemical concentration, and time. Tadpoles exposed to high concentrations of carbaryl had lower survival rates at 27°C than at 17°C and 22°C, but at low chemical concentrations, tadpoles survival was unaffected by temperature (i.e.
temperature treatment alone was not lethal). At high temperatures, a lower concentration of carbaryl is needed to induce mortality. Because many amphibians breed and develop in waters that commonly reach 27ºC or greater, carbaryl may be more potent in the environment than expected (Boone and Bridges 1999). Carbaryl is absorbed by the body more rapidly at high temperatures, but metabolism also increases with temperature, therefore the chemical may be broken down and excreted more rapidly as well (Boone and Bridges 1999). Furthermore, although carbaryl toxicity increases with temperature, the chemical also breaks down more quickly in the aquatic environment at higher temperatures, thus amphibians may be exposed to a more toxic insecticide but for a shorter period of time. This study demonstrated the importance of testing chemicals at a range of temperatures.

Relyea and Mills (2001) exposed *Hyla versicolor* tadpoles to sublethal levels of carbaryl and chemical cues of a predator, *Ambystoma maculatum*, enclosed within a cage. Carbaryl concentrations (in the absence of predators) of only 0.09 and 0.05 mg/L caused high mortality within one week, most mortality occurring after 5 days. At 0.09 mg/L, survival declined to about 8% by day 8, regardless of predator treatment. At 0.05 mg/L, carbaryl caused survival to decline to 40% in the absence of predators, and to 3% in the presence of predators. At concentrations of 0.07, 0.14, 0.27, and 0.54 mg/L, predator cues caused carbaryl to be four times more lethal; survivorship averaged 32% across all treatments. The interaction of carbaryl and predator cues induced mortality, significantly reduced tadpole activity, and reduced tadpole growth by 50% compared to controls. So, very low concentrations of carbaryl can drastically affect amphibian behavior, growth, and survival (Relyea and Mills 2001).
Relyea (2003) then exposed anuran species to five concentrations of carbaryl and predator cues (i.e. caged adult newt, Notophthalmus viridescens) to test for synergistic interactions (i.e. indirect effects) and estimate LC50 values in the presence and absence (see Direct Effects above) of predator cues. LC50 values in the presence of predator cues for *Rana pipiens*, *Rana clamitans*, *Rana catesbeiana*, *Bufo americanus*, and *Hyla versicolor* were 2.2 mg/L, 1.1 mg/L, 1.0 mg/L, 3.4 mg/L, and 2.5 mg/L, respectively.

Synergistic interactions between carbaryl and predator cues were not detected in *Rana sylvatica* and *Hyla versicolor*. In fact, wood frogs (*Rana sylvatica*) experienced significant mortality from predator cues in the absence of carbaryl; thus the LC50 estimate was not determined for indirect effects on this species (Relyea 2003). For *Rana pipiens* and *Bufo americanus*, synergistic interactions were apparent early in the experiments at high concentrations, but synergies disappeared because carbaryl alone eventually killed all the tadpoles. Highly synergistic interactions persisted in *R. clamitans* and *R. catesbeiana*—predator cues caused carbaryl to be up to 8 and 46 times more deadly in *R. clamitans* and *R. catesbeiana*, respectively (Relyea 2003).

A noteworthy observation is that synergies were detected in two Missouri populations of *H. versicolor* using chemical cues of *Ambystoma maculatum* (Relyea and Mills 2001), but were not found in the Pennsylvania population, using *N. viridescens* cues (Relyea 2003). Therefore, either different predators cause different synergistic effects or different populations have different susceptibilities to the interactions of carbaryl and predator cues (Relyea 2003). Because there are significant potential interactions between chemical contaminants and predatory stress, further investigation must evaluate the synergistic relationships of the stressors.
Field Studies

Laboratory tests are valuable in assessing the direct toxicity of chemical to an amphibian species; however the results of these tests may not accurately represent the effects of chemical contamination in the environment (Relyea 2005). For instance, laboratory studies have shown negative direct effects of carbaryl on anuran metamorphosis and survival (e.g., reduced survival and smaller size at metamorphosis) (Bridges 1997, 1999). In contrast, mesocosm studies, which test the effects of the contaminant on experimental aquatic communities, using a natural food web, have shown positive indirect effects on anuran metamorphosis and survival (e.g., increased survival and larger size at metamorphosis) resulting from increased periphyton (i.e. food) abundance and decreased predation (i.e. reduced predator survival) (Mills & Semlitsch 2004; Boone & Semlitsch 2001, 2002, 2003). In these studies, the indirect benefits to the anurans that result from changes in the aquatic community prevailed over the direct consequences of chemical toxicity to the species. Mesocosm studies that replicate contamination events on a natural aquatic ecosystem by utilizing natural biotic factors such as plankton (i.e. food), natural predators and competitors, as well as abiotic factors such as temperature, pond drying (i.e. hydroperiod), and pH, are therefore credited more suitable to represent the effects of chemical exposure on a species in its natural environment (Bridges and Semlitsch 2000, Mills 2002, Boone and Bridges-Britton 2006).

*Notopthalmus viridescens* larvae, although not directly affected by a concentration of carbaryl, suffer mass mortality by carbaryl contamination of the aquatic environment (Mills 2002; Mills and Semlitsch 2003). When the plankton that they depended upon for
nourishment were exterminated by the chemical, the salamanders died of starvation (Mills 2002; Mills and Semlitsch 2003). Similar effects have been observed for *Ambystoma maculatum* growth and survival (Boone and James 2003; Boone et al. 2007). Thus, it is essential to understand the effects of the chemical on other organisms that exist within the amphibian’s natural environment. “When toxicity studies are embedded in the nexus of interactions that compose natural food webs, we can arrive at very different interpretations due to the prevalence of both direct and indirect effects” (Relyea 2005).

It is essential to include natural factors such as competition, predation, and pond hydroperiod (Boone and James 2003) as they are important in community processes (Semlitsch et al. 1996), and understanding the effects of a chemical is impossible without considering the complex environments that are also altered with chemical exposure (Boone and James 2003). Therefore, not only must scientists consider appropriate chemical concentrations, but it is also imperative to consider natural biological conditions when determining the impacts of insecticides on amphibians (Relyea 2005).

**Multiple Stressors**

Environmental stressors such as increased UV-radiation and pathogens can interact with pesticides to have synergistic effects on amphibian species by increasing chemical toxicity or by increasing the vulnerability of the victim to other factors; thus, it is necessary for studies to examine the role of multiple stressors in amphibian communities (Boone and Bridges-Britton 2006). A single stressor is unlikely to act alone in an aquatic ecosystem; consequently the effects of the stressor may be dependent upon its interactions with other environmental factors (Boone et al. 2007).
Combinations of contaminants in the presence of other stressors have been shown to have additive (Boone and James 2003) and synergistic (Boone et al. 2005, Relyea 2004, Hayes et al. 2003) interactions, although multiple, sublethal chemical stressors may be no more damaging than the presence of a single chemical (Relyea 2005, Boone and Bridges-Britton 2006, Puglis and Boone 2007). Combinations of multiple chemical stressors in the environment could likely increase amphibian susceptibility to other environmental factors, therefore causing population declines, (Carey et al. 1999, Boone et al. 2007) but the combination of stressors may not produce effects that are predictable by the effects observed by a single stressor (Boone et al. 2008).

Amphibians exposed to one agrichemical are likely to be exposed to more than one. Crop maintenance, in farming practices as well as general lawn and garden care, generally requires the use of fertilizers in addition to herbicides and insecticides. Boone et al. (2005) found that carbaryl in the presence of ammonium nitrate, directly affected Rana clamitans by creating greater metabolic demands, leaving less energy for feeding or food assimilation thereby, reducing growth and development. Boone and Bridges-Britton (2006), however, reported no additive effects associated with the combination of carbaryl and ammonium nitrate exposure on Hyla versicolor.

Boone et al. (2007) tested the interactions of (2.5 mg/L) carbaryl and (10 mg/L) ammonium nitrate fertilizer on an experimental aquatic community comprised of Bufo americanus tadpoles, Rana sphenoecephala tadpoles, and Ambystoma maculatum salamander larvae. Overwintered Rana catesbeiana bullfrogs served as competitors with the tadpoles and bluegill Lepomis macrochirus were top predators. Boone et al. (2007) did not observe negative synergistic interactions between the chemicals. In fact, the
insecticide and the fertilizer increased periphyton (i.e. food) abundance which enhanced anuran survival and increased mass at metamorphosis. *Rana sphenoecephala* exposed to either bullfrogs or bluegill had decreased time to metamorphosis, while tadpoles exposed to both bullfrogs and bluegill increased time to metamorphosis.

*Ambystoma maculatum* were affected by carbaryl treatment and the interaction of carbaryl and bluegill which reduced cladoceran (i.e. food) abundance thereby reducing salamander mass, increasing time to metamorphose, and causing mortality to the salamanders in the aquatic community (Boone et al. 2007). Carbaryl exposure likely compromises growth, reproduction, and survival in *A. maculatum* in the environment. Boone et al. (2007) suggests that *A. maculatum* may be most able to tolerate additional biotic stress in the absence of chemicals.

Boone et al. (2001) and Boone and Bridges (2003) exposed *Rana clamitans* tadpoles to sublethal concentrations of carbaryl to test the effects of multiple exposures to the insecticide and timing of exposure. The number of times tadpoles were exposed to carbaryl significantly increased the rate of metamorphosis, and decreased metamorph size (Boone et al. 2001, Boone and Bridges 2003). Carbaryl caused mortality to tadpoles exposed to carbaryl one time late in development, while tadpole survival was greater to tadpoles exposed three times: one time during early, mid- and late development (Boone and Bridges 2003). Boone (2008) asserted that pesticides with the same mode of action (e.g., cholinesterase inhibitors) are less likely to produce additive effects from multiple exposures in comparison to chemicals with different modes of action.

The indirect effects of carbaryl exposure at concentrations expected to occur in the environment may have positive effects on anurans due to increased food resources
(Boone et al. 2008); however the effects of an additional stressor in the aquatic community could reduce anuran diversity. For instance, Boone and Semlitsch (2003) found that carbaryl enhanced the size and development of bullfrogs (*Rana catesbeiana*), while the chemical induced predator (*Notophtalmus viridescens, Lepomis macrochirus*, and *Orconectes sp.*) mortality presumably due to reduction in zooplankton food resources. Bullfrogs, especially introduced populations, can be damaging to other amphibian species by acting as competitors, predators, or disease vectors (Boone et al. 2008). Carbaryl exposure could allow bullfrogs to invade new communities by reducing or eliminating predators, which could have negative consequences for other anurans in the community (Boone and Semlitsch 2003, Boone et al. 2008). These data could help explain bullfrog success in “highly managed areas,” (Boone and Semlitsch 2003, Boone et al. 2008).

Interactive effects of UV-B radiation and chemical stressors are essential to toxicity studies because UV-radiation alone can threaten amphibian survival and in the presence of a pesticide, it may increase chemical toxicity. Zaga et al. (1998) tested the interactive effects of UV radiation and carbaryl treatment on embryos and tadpoles of *Hyla versicolor* and *Xenopus laevis*. Synergism between UV radiation and carbaryl appeared to occur by photomodification of the carbaryl molecule in water (Zaga et al. 1998). The study found that carbaryl absorbs UV-A as well as UV-B radiation, and UV-A also photoactivates the chemical, but to a lesser extent than with UV-B.

While 100% mortality occurred in *X. laevis* embryos exposed to carbaryl and high UV-B radiation simultaneously, mortality was not observed in *X. laevis* embryos previously exposed to carbaryl and subsequently exposed to UV-B radiation; therefore
the photochemical transformation of carbaryl likely produced the synergistic effects observed in all experiments (Zaga et al. 1998). A ten-fold increase in toxicity resulted from photoactivation of carbaryl by approximately 1.5% ambient UV-B (Blaustein et al. 2003). Increased swimming activity, followed by decreased locomotor activities, and developmental abnormalities, such as lateral flexure of the tail, were also observed in *X. laevis* and *H. versicolor* tadpoles exposed to UV-B radiation and carbaryl concentrations of 0.86, 1.24, and 1.76 mg/L (Zaga et al. 1998). Hence, the acceptable level of carbaryl in the environment, using *H. versicolor* as the model species, may be much below 1.24 mg/L.

The interaction between pesticides and pathogens is also important to the study of amphibian population declines. These chemicals may inhibit immune defenses and increase susceptibility to disease (Davidson et al. 2007). Puglis and Boone (2007) exposed *Rana catesbeiana* embryos and tadpoles to the pathogen, *Saprolegnia ferax*, and sublethal levels of carbaryl and ammonium nitrate under laboratory conditions. Significant interactive effects between the pathogen, the fertilizer, and the insecticide were not observed, although similar concentrations of carbaryl were found to interact with other amphibian species (Puglis and Boone 2007).

Davidson et al. (2007) exposed post-metamorphic juvenile *Rana boylii* to the chytid fungus (*Batrachochytrium dendrobatidis*) and sublethal levels of carbaryl. Although carbaryl exposure inhibited peptide defenses in *Rana boylii* juveniles, no significant direct effects on survival or growth were apparent from the interaction between chytid and carbaryl exposure (Davidson et al. 2007). Davidson et al. (2007) reported that the results could be due to either species or localized resistance to the strain
of chytid used in the study and that *R. boylii* may be more susceptible to different strains of chytid than the one in this study. Further studies are needed to examine the interactive effects of pathogens and carbaryl.

Amphibians are continuously responding to multiple abiotic (e.g., temperature, UV-radiation, pond drying) and biotic (e.g., food resources, predation, competition, density, pathogens) stressors. The complex interactions among these factors thus determine amphibian survival. In order for conclusions to be made concerning the relative impacts of agrichemicals on amphibian populations, further research must be employed to examine the interactive effects of agrichemicals and environmental stressors on aquatic communities. Stressors relating to current global change and amphibian declines (e.g., UV-B radiation, temperature change, pathogens contributing to EIDs) are critical to further studies.

**Conclusion**

The increased use of chemical pesticides is believed to contribute to the decline in amphibian biodiversity. It is necessary to determine how the agrichemicals are impacting amphibian populations in order to slow the rate of amphibian declines (i.e. reduce human impacts from farming practices) and prevent irreversible damage to ecosystems. If pesticides are contributing to declines, it is not likely due to direct lethal effects, but sublethal effects and interactions with other stressors (Davidson et al. 2007). Single factor studies may be insufficient to determine the widespread phenomena of amphibian declines (Boone and James 2003). Because amphibian declines are generally believed to be caused by multiple stressors, it is logical to conclude that better understanding of the
declines will therefore be found through the examination of the interactive effects of those stressors.

In combination with multiple stressors, toxicology studies must also include sublethal concentrations of the pesticides, which could interfere with population dynamics and produce indirect effects caused by change in the aquatic community. Study of these effects are essential to pesticide toxicity research and should be utilized in chemical regulation. Also, in order to gain understanding about local adaptation and persistence of species in contaminated environments, it is necessary to determine the presence of genetic variation for tolerance in populations (Semlitsch et al. 2000). Toxicology studies that assess the susceptibility to contaminants and other stressors in the environment among different species and populations are necessary for monitoring populations and could also aid in pesticide regulation. Additional studies on the additive effects of multiple chemical exposures, timing (i.e. life stage) and frequency of exposures, and chemical mixtures are necessary as well to link toxicology studies with amphibian exposure in the environment.

Long-term toxicology studies and monitoring programs are critical to future research. It is important to study multiple chemical exposures in aquatic communities for multiple generations to gain understanding of population dynamics. Long-term studies examining the shifts in community structure after the disappearance of key species could also provide beneficial data. Finally, further studies to assess changes in species habitat use as a result of contamination in the environment are needed. Storfer (2003) stressed the use of monitoring programs that are statistically sensitive to changes in amphibian populations. By determining the distribution and abundance of amphibian populations,
researchers will be more capable of assessing the threat and the extent of population declines. Conservation efforts should also be made to monitor the landscape to determine the extent and abundance of suitable habitats (Storfer 2003). Finally, molecular genetic techniques should be employed to monitor demographics and identify significant events such as population fragmentation and hybridization (Storfer 2003). Together, these techniques should help to recognize major issues contributing to amphibian declines, identify which species are most threatened, and aid in slowing the rate of amphibian species loss. Furthermore, by employing the techniques described above, researchers may be able to recognize patterns, make generalizations, and create a more standard mechanized approach to understanding amphibian declines.
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