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Effects of herbicides and the chytrid fungus *Batrachochytrium dendrobatidis* on the health of post-metamorphic northern leopard frogs (*Lithobates pipiens*)

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ABSTRACT

Effects of exposure to contaminants such as pesticides along with exposure to pathogens have been listed as two major contributors to the global crisis of declining amphibian populations. These two factors have also been linked in explanations of the causes of these population declines. We conducted a combined exposure experiment to test the hypothesis that exposure to two agricultural herbicides would increase the susceptibility of post-metamorphic northern leopard frogs (*Lithobates pipiens*) to the amphibian fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). We assessed the independent and interactive effects of these exposures on the health and survival of the frogs. Wild-caught frogs underwent a 21-day exposure to a nominal concentration of either 2.1 μg/L atrazine (Aatrex® Liquid 480) or 100 μg a.e./L glyphosate (Roundup® Original), followed by *Bd*, and then were observed until 94 days post-initial exposure to the herbicides. Actual levels of atrazine were between 4.28 ± 0.04 μg/L and 1.70 ± 0.26 μg/L while glyphosate degraded from 100 μg a.e./L to approximately 7 μg a.e./L within 6 days of initial exposure to the herbicides. Compared to controls, the glyphosate formulation reduced the snout-vent length of frogs during the pesticide exposure (at Day 21), and the atrazine formulation reduced gain in mass up to Day 94. No treatment affected survival, splenosomatic or hepatosomatic indices, the densities and sizes of hepatic and splenic melanomacrophage aggregates, the density and size of hepatic granulomas, proportions of circulating leucocytes, the ratio of neutrophils to lymphocytes, or the ratio of leucocytes to erythrocytes. Histological assessment of samples collected at Day 94 revealed no evidence of *Bd* infection in any *Bd*-exposed frogs, while real-time PCR detected only one case of light infection in a single atrazine- and *Bd*-exposed frog. Frogs exposed to *Bd* shed their skin significantly more frequently than *Bd*-unexposed frogs, which may have helped them resist or clear infection, and could explain why no interaction between the herbicides and *Bd* was detected. The results suggest that these frogs were resistant to *Bd* infection and that pre-exposure to the herbicides did not alter this resistance. The effects seen on the growth following herbicide exposure is a concern, as reduced growth can lower the reproductive success and survival of the amphibians.

1. Introduction

Amphibian populations have experienced severe declines over the past three decades and more than one-third of amphibian species are now threatened with extinction (Stuart et al., 2004). The disease chytridiomycosis, caused by infection by the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter "*Bd*"), has been identified as a proximate cause of both amphibian population declines and species extinctions, but the factors underlying its recent emergence and varying virulence remain under investigation (Fisher et al., 2009). With respect to virulence, one hypothesis states that an interaction between environmental contaminants, such as pesticides, and *Bd* may contribute to susceptibility to infection (Carey et al., 1999; Parris and Baud, 2004; Davidson et al., 2007). The prevalence of pesticides in aquatic environments combined with observations that amphibians exhibit immune suppression and increased parasitism following pesticide exposure (e.g. Taylor et al., 1999; Kiesecker,

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2002; Christin et al., 2003, 2004) suggest that environmental pesticide exposure may influence the dynamics of amphibian disease, including chytridiomycosis.

Northern leopard frogs (*Lithobates pipiens*; formerly *Rana pipiens*) are exposed to pesticides and *Bd* throughout their range (Ouellet et al., 1997; Smith and Keinath, 2007; Tennessen et al., 2009). Although widely distributed across North America (Voordouw et al., 2010), certain northern leopard frog populations are threatened and the species' range is contracting (Smith and Keinath, 2007; Voordouw et al., 2010); in Canada, the western populations are listed as endangered (COSEWIC, 2009). However, eastern populations of leopard frogs appear to exhibit resistance to *Bd* and may serve as a reservoir of infection within amphibian communities (Ouellet et al., 2005; Longcore et al., 2007; Woodhams et al., 2008). Other leopard frog populations appear more susceptible to *Bd*, as chytridiomycosis was the cause or suspected cause of mass die-offs (Carey et al., 1999; Green et al., 2002; Voordouw et al., 2010) and individual mortality (Adama et al., 2004).

The objective of the current study was to investigate whether the resistance that some amphibians exhibit toward *Bd* is compromised by exposure to environmental contaminants including pesticides. Therefore, we investigated whether post-metamorphic northern leopard frogs from eastern Canada could become susceptible to clinical *Bd* infections following exposure to pesticides. To accomplish this, we performed a combined exposure experiment, assessed the independent and interactive effects of common pesticides and *Bd* on individual frog health and survival, and tested the hypothesis that exposure to the herbicides would increase susceptibility to infection by *Bd*. The frogs were exposed to sublethal concentrations of the herbicides Aatrex® Liquid 480 or Roundup® Original, then *Bd*, and subsequently observed until 94 days post-initial exposure to the herbicides. The Aatrex® formulation contains the active ingredient atrazine, which has been linked to depressed immune systems in amphibians (Christin et al., 2003, 2004; Forson and Storfer, 2006; Rohr et al., 2008a, 2008b). The Roundup® formulation contains the active ingredient glyphosate and the surfactant polyethoxylated tallowamine (POEA) (Giesy et al., 2000), both of which have been widely investigated for their toxic effects in amphibians (e.g. see review in Govindarajulu, 2008) but less so for their potential immunomodulating effects (Rohr et al., 2008a). We evaluated survival, growth (gain in mass and snout-vent length), measures of condition (hepatosomatic index, densities and mean sizes of hepatic and splenic melanomacrophage aggregates, density and mean size of hepatic granulomas, and neutrophil/lymphocyte ratios), markers of immune function (splenosomatic index, differential leucocyte counts, ratio of leucocytes to erythrocytes), and susceptibility to *Bd* using standard molecular and histological techniques (Hyatt et al., 2007).

2. Materials and methods

2.1. Ethics statement

This study was conducted in accordance with national and institutional guidelines for the protection of animal welfare, as outlined by the Canadian Council on Animal Care (CCAC, 1993) and the Aquatic Ecosystem Protection Research Division Animal Care Committee (St. Lawrence Centre, Environment Canada).

2.2. Animals

In August 2007, recently-metamorphosed northern leopard frogs were captured on land, near a pond located in a wildlife reserve near Boucherville, Quebec (45°38′06″N 73°26′06″W). Immediately after their capture, a skin swab was taken from each of the 20 randomly selected individuals to test for infection with *Bd* using methods described by Kriger et al. (2006). Each swab was placed in a sterilised Eppendorf tube and sent within 24 h to the British Columbia Ministry of Agriculture's Animal Health Centre (Abbotsford, BC, Canada), where all swabs were tested for *Bd* using a real-time PCR assay designed to target the ITS1 gene of the fungus (Julie Bidulka, personal communication). Water samples taken from the pond were analysed for 39 pesticides commonly used in eastern Canadian agriculture, including atrazine and glyphosate. Pesticide analyses were conducted at the Centre d'expertise en analyse environnementale du Québec (CEAEQ, Quebec City, QC, Canada).

The frogs were transported to the laboratory where they were isolated in individual 1.84 L plastic containers. The containers held 50 mL of dechlorinated, UV-treated water, and two platforms that permitted the frogs to exit the water. The frogs were acclimated to a 16 h:8 h light:dark photoperiod and a room temperature of 21 ± 1.3 °C over a period of eight weeks.

During the experiment, each frog was housed in a 1 L mason jar containing 50 mL of the appropriate treatment liquid for the corresponding phase of the study (see below). The mouth of each jar was covered with fibreglass screening secured with the jar's screw-on ring to prevent escape. With the exception of the period during which the frogs were exposed to *Bd*, the jars were placed on a slant to allow the frogs to enter and leave the liquid at will, given the semi-terrestrial nature of the species post-metamorphosis.

During the acclimatisation and experimental periods, the water or exposure solution in each jar was changed daily, as it rapidly became fouled. The location of individual jars was randomly interchanged on a weekly basis by blindly drawing labels out of a container, to minimise effects of uneven lighting or temperature within the laboratory. The frogs were fed one to two crickets (Mirdo Importations Canada, Inc., Montreal, QC, Canada) every 2 days. Once a week, the crickets were pre-dusted with phosphorus-free $CaCO_3$ powder (Mirdo Importations Canada, Inc., Montreal, QC, Canada) to prevent nutrient deficiencies in the frogs.

At various stages of the experiment, frogs were killed by immersion in a solution of 0.8% buffered tricaine-methanylsulfonate (MS-222) in distilled water (Syndel Laboratories Ltd, Vancouver, BC, Canada).

2.3. Chemicals and reagents

Aatrex® Liquid 480, containing the active ingredient atrazine, was purchased from Syngenta Crop Protection Canada Inc. (Guelph, ON, Canada). Roundup® Original, containing the active ingredient glyphosate, was purchased from Monsanto Canada Ltd. (Winnipeg, MB, Canada). All reagents used in the study were purchased from Fisher Scientific (Ottawa, ON, Canada) unless otherwise noted.

2.4. Aatrex® Liquid 480 and Roundup® Original exposures

A time-frame of 21 days was selected for the chemical exposures to mimic realistic exposure times in southwestern Quebec (Giroux et al., 2006). A concentration of 2.1 μg/L was selected for atrazine because it reflected concentrations measured in summer surface waters within the same region (Gendron et al., 2003). A concentration of 100 μg a.e./L (a.e.=acid equivalents of glyphosate) was selected for glyphosate, based on average and maximum concentrations (600 and 1800 μg a.e./L, respectively) reported in a widely-cited ecotoxicological risk assessment study (Giesy et al., 2000). Although this was well above concentrations measured in surface waters of the study area (< 0.8 μg a.e./L, Giroux et al., 2006), concentrations of glyphosate in surface waters, including vernal pools, streams and forest wetlands, which are important amphibian habitats, can reach levels as high as 1950 μg a.e./L (Feng et al., 1990; Giesy et al., 2000; Thompson et al., 2004; Scribner et al., 2007; Battaglin et al., 2009).

One day prior to the start of the experiment, two chemical stock solutions were prepared, each at $1000\times$ the target exposure concentrations for atrazine and glyphosate, by dissolving the water-soluble end-use herbicide formulations Aatrex® Liquid 480 and Roundup® Original, respectively, in deionized water. Stock solutions were stored at 4 °C in sealed 2 L amber flasks that were held in light-proof boxes to prevent photodegradation. During the pesticide exposure phase of the experiment (Days 1–21), aliquots of the stock solutions were diluted daily with dechlorinated water, and used to replace the pesticide solutions in jars holding the frogs being exposed.

At Day 20 of the experiment, two samples of freshly diluted stock solution of each herbicide were collected. These samples were later analysed to determine the initial herbicide concentration in the exposure solutions used for the daily exposures during the pesticide exposure phase. At Day 21 of the experiment, immediately following the final exposure of the frogs to the herbicides, water samples from six randomly-chosen exposure vessels per pesticide group were collected for analysis. The atrazine samples (the Day 20 freshly-diluted stock solution representing the exposure solution, along with the Day 21 water samples from the exposure vessels) were immediately shipped to the National Laboratory for Environmental Testing (NLET, Environment Canada, Burlington, ON, Canada) for analysis. The corresponding glyphosate samples were stored in sealed amber bottles and refrigerated at 4 °C for three months prior to being sent for analysis by AXYS Analytical Services Ltd. (Sidney, BC, Canada). The original concentrated stock solutions were not analysed.