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

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Abstract

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

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Numerous studies have shown that pesticides can adversely affect amphibian health and suppress immune function, making them more susceptible to pathogens and disease. This study assessed the independent and combined effects of exposure to two agricultural herbicides and the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) on the health and survival of post-metamorphic northern leopard frogs (*Lithobates pipiens*). Wild-caught frogs were exposed to the herbicides atrazine (Aatrex® Liquid 480) or glyphosate (Roundup® Original) for 21 days and subsequently challenged with *Bd*. The glyphosate formulation significantly reduced growth compared to controls during the pesticide exposure. The atrazine formulation significantly reduced gain in mass at 94 days post initial exposure to the pesticides. No treatment significantly affected survival, the numbers of leucocytes, the hepatosomatic index, the splenosomatic index, the numbers and sizes of melanomacrophage aggregates in the liver or spleen, or the numbers and sizes of granulomas in the liver. Histological tests revealed no evidence of *Bd* infection in any *Bd*-exposed frogs, while molecular tests (real-time PCR) detected only one case of light infection (1.6 DNA copies) in an 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. Overall, the results suggest that these frogs were resistant to *Bd* and that pre-exposure to the pesticides did not alter this resistance. However, reduced growth can lower the reproductive success and survival of amphibians, and therefore, exposure to the pesticides may contribute to population reductions in leopard frogs.

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Introduction

Severe population declines have been observed among amphibians for over the past three decades and over 30% of amphibian species are now threatened with extinction (Stuart *et al.* 2004). This is alarming because amphibians form a vital component of many ecosystems, and their amphibious nature, eggs without shells and permeable skin make them good indicators of environmental stress (Blaustein and Kiesecker 2002). Infectious diseases may play a significant role in many of these declines (Green *et al.* 2002; Daszak *et al.* 2003), but pesticides and other environmental contaminants may also play a role by enhancing the susceptibility of amphibians to opportunistic pathogens (Dazsak *et al.* 1999).

Numerous studies have demonstrated that pesticides can directly alter amphibian growth, development, behaviour, reproduction and survival (Carey and Bryant 1995). Immunosuppressive effects due to pesticides have also been documented (Taylor *et al.* 1999; Kiesecker 2002; Gendron *et al.* 2003; Gilbertson *et al.* 2003; Christin *et al.* 2004; Albert *et al.* 2007; Rohr *et al.* 2008b). The use of pesticides in agriculture and silviculture exposes amphibians to these chemicals when they breed and develop in open ponds and forest wetlands (Berrill *et al.* 1997; Battaglin *et al.* 2009). Given the widespread occurrence of pesticides as environmental pollutants (Relyea and Hoverman 2006), it is possible that they are contributing significantly to amphibian declines not only through direct toxic effects, but also as drivers of epizootic disease (Davidson *et al.* 2007). This study addressed these concerns by exposing frogs to two widely used pesticides and subsequently to an important fungal pathogen associated with global amphibian population declines.

Pesticides

This study used the formulated herbicides Aatrex® Liquid 480 (atrazine) and Roundup® Original (glyphosate), which are commercially available for agricultural use. Vision® herbicide is chemically equivalent to Roundup® Original, but is used in the forestry industry (Wojtaszek *et al.* 2004).

Aatrex® Liquid 480 mainly contains atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) as its active ingredient, along with undisclosed related triazines and 1,2-benzisothiazolin-3-one at 0.019% as a preservative. Atrazine is one of the most heavily used agricultural herbicides in the world (Ackerman 2007). It is used predominantly for broad-leaf weed control in corn and sorghum production (Solomon *et al.* 1996; Rohr *et al.* 2004). Its half-life in aquatic environments ranges from 3.2 days to 7 or 8 months (CCME 1999), depending factors that include temperature, pH, light, sediment type and concentration (Solomon *et al.* 1996). Atrazine inhibits photosynthesis in plants by blocking electron transport in photosystem II, and therefore should be much less toxic to animals than plants (Eisler 1989; Solomon *et al.* 1996). In fact, a number of studies conducted to assess the impact of atrazine on frog survival concluded that concentrations found to have adverse effects are considerably greater than those found or predicted to occur in surface waters in North America (Solomon *et al.* 2008). However, there is evidence suggesting that atrazine acts as an endocrine disruptor in amphibians, altering normal rates of growth and development, and inducing gonadal abnormalities, even in small doses (Tavera-Mendoza *et al.* 2002a, 2002b; Hayes *et al.*, 2002, 2003,

2006; Brown Sullivan and Spence 2003; Rohr *et al.* 2004, 2006; Coady *et al.* 2004; Freeman and Rayburn 2005; Houck and Sessions 2006; Floyd *et al.* 2008; Relyea 2009; Langlois *et al.* 2010). Atrazine may affect sexual differentiation by increasing levels of the enzyme aromatase, which converts androgen to estrogen, and it may affect growth and development by altering levels of thyroid hormones (thyroxine and triiodothyronine) and corticoserone (Tavera-Mendoza *et al.* 2002a, 2002b; Brown Sullivan and Spence 2003).

Atrazine also appears to act as an immunosuppressant in amphibians, increasing their susceptibility to parasites and disease (Kiesecker 2002; Forson and Storfer 2006; Houck and Sessions 2006; Koprivnikar *et al.* 2007; Rohr *et al.* 2008b; Rohr and McCoy 2010). As evidence of this, populations of tiger salamanders (*Ambystoma tigrinum*) in Arizona suffered disease outbreaks caused by the *A. tigrinum* virus (ATV) following widespread local use of the herbicide (Forson and Storfer 2006). Laboratory-bred progeny of the infected populations exhibited significantly reduced peripheral leukocyte counts following experimental exposure to 16 or 160 μg/L atrazine compared to those exposed to 0 or 1.6 μg/L, and those exposed to 16 μg/L atrazine were also shown to be more susceptible to ATV infection (Forson and Storfer 2006). In another study, white blood cells (WBC) of adult northern leopard frogs (*Lithobates pipiens*) exposed to 0.1 μg/L atrazine demonstrated reduced migratory and phagocytic activity following thioglycollate stimulation (Brodkin *et al.* 2007). Still other studies have linked exposure to atrazine at concentrations between 30 and 250 μg/L with reduced numbers of lymphocytes in the blood (Hecker *et al.* 2005), increased susceptibility to infection by trematodes (Koprivnikar *et al.* 2007; Rohr *et al.* 2008a), or both (Kiesecker 2002). More

recently, microarray studies demonstrated that exposure of larval African clawed frogs (*Xenopus laevis*) to 400 µg/L atrazine down-regulated genes associated with immunity and other body functions (Langerveld *et al.* 2009). These included genes involved with epidermal secretions that protect amphibians against viruses, bacteria and other pathogens which attack the skin (Langerveld *et al.* 2009).

Roundup® Original and Vision® herbicides contain glyphosate [N-(phosphonomethyl) glycine] in the form of an isopropylamine salt, as their active ingredient (Giesy *et al.* 2000; Wojtaszek *et al.* 2004). They also contain polyoxyethylene tallow amine (POEA) as a surfactant to facilitate penetration through the waxy cuticle of the target plant, along with other undisclosed components (Giesy *et al.* 2000; Wojtaszek *et al.* 2004). Toxicological studies and studies describing the environmental fate of these products may report concentrations of glyphosate in terms of its acid equivalent (ae) or as the isopropylamine salt active ingredient. To facilitate comparisons between studies, it can be assumed that 1 mg of the isopropylamine salt contains 0.75 mg glyphosate ae (Giesy *et al.* 2000).

Glyphosate inhibits plant growth by blocking the production of essential amino acids through competitive inhibition of plant enzymes (Giesy *et al.* 2000). Glyphosate and its primary breakdown product aminomethylphosphonic acid (AMPA) are said to have little or no toxicity to non-target organisms (Mann and Bidwell 1999; Giesy *et al.* 2000). Nevertheless, the impacts of glyphosate and its products on freshwater ecosystems are poorly understood (Baylis 2000; Govindarajulu 2008).

Acute toxicological studies indicate that POEA increases the toxicity of formulations substantially, posing a significant risk to amphibians (Mann and Bidwell

1999, 2001; Giesy *et al.* 2000; Edginton *et al.* 2004a; Howe *et al.* 2004; Cox and Surgan 2006; Brausch and Smith 2007; Relyea and Jones 2009). Moreover, the half-life of POEA in water may be up to 20 times longer than that of glyphosate (i.e. 14 to 41 days for POEA compared to 2 to 14 days for glyphosate), suggesting that amphibians may be exposed to POEA in aquatic systems for important periods of time (Giesy *et al.* 2000; but see Wang *et al.* 2005). POEA may exhibit its negative effects on survival by disrupting the respiratory surfaces of aquatic organisms (Dinehart *et al.* 2009). Under certain conditions, Vision®, Roundup® and POEA can, respectively, be close to 100, 600 and 2000 times more toxic to frog embryos than glyphosate alone (Perkins *et al.* 2000; Edginton *et al.* 2004a). In addition to lowered survival, chronic studies examining the toxicity of POEA-containing glyphosate formulations at environmentally-realistic doses (i.e. 600 to 1800 µg ae/L for 16 to 43 days) revealed a variety of effects on amphibian larvae that include reduced length at metamorphosis, reduced mass at metamorphosis, altered time to metamorphosis, gonadal abnormalities and shortened and damaged tails (Howe *et al.* 2004; Relyea 2004; Cauble and Wagner 2005).

Little information is available on the potential immunomodulating effects of glyphosate or its formulated products on amphibians. Green frog tadpoles (*Lithobates clamitans*) exposed to 3700 µg ae/L of technical-grade glyphosate for 7 days were more susceptible to infection by the trematode *Echinostoma trivolvis* than unexposed tadpoles (Rohr *et al.* 2008a). Among fish, Nile tilapia (*Tilapia nilotica*) exhibited reduced cell-mediated and humoral immune responses when they were exposed to 1/1000 of the recommended field application rate of an undisclosed formulated glyphosate product for 96 h (El-Gendy *et al.* 1998).

It is estimated that the no-observed-effects concentration (NOEC: i.e. no effect on growth or survival) of glyphosate for chronically exposed amphibians is 740 µg ae/L (Giesy *et al.* 2000). The concentration of glyphosate set as the Canadian water quality guideline for the protection of freshwater life is 65 µg ae/L (Trotter *et al.* 1990). However, concentrations of glyphosate in surface waters, including vernal pools, streams and forest wetlands, which are important as amphibian habitats, can reach much higher levels (e.g. 1950 µg ae/L) when they are not protected by vegetated or no-spray buffer zones (Feng *et al.* 1990; Giesy *et al.* 2000; Thompson *et al.* 2004; Scribner *et al.* 2007).

Batrachochytrium dendrobatidis

Batrachochytrium dendrobatidis Longcore, Pessier and Nichols, 1999 (hereafter *Bd*) (Phylum Chytridiomycota, Class Chytridiomycetes, Order Chytridiales) is a keratinophilic chytrid fungus known to only infect amphibians, and it is recognized as a recently emerging pathogen (Berger *et al.* 1998; Daszak *et al.* 1999). Since its description less than two decades ago, *Bd* has caused mass mortalities, population declines and species extinctions among more than 200 species of amphibians globally (Berger *et al.* 2005; Fisher *et al.*, 2009). Its presence has been documented on all continents except Antarctica, where hosts are not present (Fisher *et al.* 2009), and the disease that it causes, chytridiomycosis, has been declared an internationally notifiable disease (OIE 2008). Although *Bd* is considered the most serious emerging infectious pathogen afflicting amphibians at the present time (Tennessen *et al.* 2009), it remains uncertain whether it is globally endemic and that amphibian declines associated with it have only recently been noticed; whether it is globally endemic and that its recent emergence is due to increased

pathogenicity; or whether it is rapidly spreading and infecting novel host species (Daszak *et al.* 1999).

Upon infection of post-metamorphic amphibians, flagellated *Bd* zoospores penetrate epidermal cells, mature into zoosporangia and shed new zoospores that reinfect the host or disperse into the environment (Berger *et al.* 2005). Infection may induce epidermal hyperplasia (an increase in the number of cell layers) and hyperkeratinization (an excessive development of keratin in the epidermis), resulting in chytridiomycosis (Berger *et al.* 2005). In turn, chytridiomycosis is thought to cause death by altering the normal osmoregulatory function of the skin, leading to an electrolyte imbalance and subsequent cardiac arrest (Voyles *et al.* 2009). However, the possibility that *Bd* secretes a toxin has not been ruled out (Blaustein *et al.* 2005; Rollins-Smith *et al.* 2009; Rosenblum *et al.* 2009). *Bd* is seldom lethal to pre-metamorphic amphibians because it is restricted to the oral region (Berger *et al.* 1998; but see Blaustein *et al.* 2005), which is the only site where keratin is present at this life stage (Smith *et al.* 2007). However, the survival of infected larvae may be threatened when infections induce oral malformations, as these may disrupt normal feeding behaviour and lead to reduced growth and development (Blaustein *et al.* 2005; Garner *et al.* 2009).

Susceptibility to *Bd* varies within and between species (Fisher *et al.* 2009). Variability in susceptibility may be partly explained by the composition of proteins secreted by the skin glands of amphibians (Fisher *et al.* 2009; Tennessen *et al.* 2009). These proteins, termed antimicrobial peptides (AMP), are part of an innate immune response against pathogens that attack the skin; they appear to play a primary role in the defense against *Bd* (Tennessen *et al.* 2009). Additional factors that may influence

susceptibility to *Bd* include local climate and season, history of exposure to *Bd* and other pathogens, and the strain of *Bd* involved (Berger *et al.* 2005; Fisher *et al.* 2009; Tennessen *et al.* 2009).

To date, only two studies have investigated whether a pesticide can increase the susceptibility of frogs to *Bd.* In the first, the survival of post-metamorphic foothill yellow-legged frogs (*Rana boylii*) exposed to the insecticide carbaryl, followed by *Bd*, was not affected by either stressor or their interaction, but growth was greatly reduced by *Bd* infection (Davidson *et al.* 2007). In the second study, American bullfrog tadpoles (*Lithobates catesbeianus*) exposed to *Bd* appeared to clear or avoid infection when they were previously exposed to the insecticide malathion, suggesting that the pesticide boosted the immune response of the tadpoles to the fungus (Charbonneau 2006). No studies have investigated the combined effects of atrazine or glyphosate coupled with *Bd* on amphibian health and survival.

Lithobates pipiens

Northern leopard frogs are native to North America, existing in most of the northern states of the United States, and far north in most Canadian provinces (Smith and Keinath 2007). The species remains abundant in many regions, but its range is contracting and it is threatened in other regions (Longcore *et al*, 2007; Smith and Keinath 2007; COSEWIC 2009). A recent taxonomic revision of the Amphibia (Frost *et al.* 2006) placed them and related species (previously genus *Rana*) in the genus *Lithobates.*

Post-metamorphic northern leopard frogs served as an ideal experimental animal because they inhabit a variety of habitats, including agricultural and forest wetlands,

where pesticides are commonly used (Ouellet *et al.* 1997; Smith and Keinath 2007). In addition, they are exposed to *Bd* throughout their range (Tennessen *et al.* 2009). While certain populations have suffered epizootic chytridiomycosis (Carey *et al.* 1999; Green *et al.* 2002; Tennessen *et al.* 2009), others exhibit resistance to *Bd*, and there is evidence that some eastern populations serve as a reservoir of infection within amphibian communities (Ouellet *et al.* 2005; Longcore *et al.* 2007; Woodhams *et al.* 2008). It is unknown whether this inter-population diversity is due to past encounters with pathogens, natural selection, genetic drift, phenotypic plasticity or abiotic factors (Tennessen *et al.* 2009). Therefore, knowledge of whether exposure to pesticides alters the susceptibility of leopard frogs to *Bd* could help efforts to protect threatened populations. In addition, it could be useful to future studies that would investigate similar questions using amphibian species that are more vulnerable to *Bd*.

Effects on survival, growth, animal health and immune function

In toxicological studies, a biomarker is a characteristic objectively measured as an indicator of normal biologic or pathogenic processes. To assess the effects of the experimental exposures on the frogs, I measured survival, growth, and various biomarkers of animal health and immune function. The biomarkers included hepatosomatic index, splenosomatic index, the densities and sizes of melanomacrophage aggregates in the liver and spleen, the densities and sizes of hepatic granulomas, differential WBC counts, the ratio of WBCs to red blood cells (WBC/RBC), and the ratio of neutrophils to lymphocytes (N/L).

Growth is commonly measured in toxicological studies. Pesticides may affect growth if normal endocrine functions are disrupted (Hayes *et al.* 2006; Mann *et al.* 2009). Alternatively or additionally, pesticides may reduce the feeding rates or food assimilation efficiency of animals and thereby impose an energy cap on growth (Mann *et al.* 2009). Pesticides may also force organisms to divert energy from growth to detoxification or tissue repair processes (Schlenk *et al.* 2008; Mann *et al.* 2009). In any case, a general underlying assumption in toxicological studies is that growth will be altered by exposure to toxicants (Schlenk *et al.* 2008).

Pathogens may also affect growth by various means. *Bd* has been reported to reduce growth in experimentally exposed post-metamorphic foothill yellow-legged frogs (Davidson *et al.* 2007). While the cause of reduced growth was not determined in that study, it is widely assumed that mounting an immune response is energetically costly, and that trade-offs with other energy-demanding processes such as growth are required to support immunocompetence (Lochmiller and Deerenberg 2000).

Organosomatic indices are common condition indices used to assess adverse impacts of contaminants in fish (Schlenk *et al.* 2008), although they are less frequently used for amphibians. The hepatosomatic index (HSI), which is the ratio of liver weight to body weight, is a preferred measure because the liver is central in detoxification processes (Schlenk *et al.* 2008). HSI is frequently elevated in fish exposed to contaminants (Yang and Baumann 2006), which can indicate induction of biotransformation enzymes in the liver, impairment of its excretory or nutrient redistribution functions, or an inflammatory response to hepatic cell damage (Crawshaw and Weinkle 2000; Hinton *et al.* 2008; Froese *et al.* 2009). Amphibians experiencing

immunosuppression following exposure to pesticides may also exhibit hepatomegaly if they are infected by opportunistic bacteria (e.g. *Aeromonas hydrophila*) that are commonly present on the skin and in the alimentary tract (Cunningham *et al.* 1996; Taylor *et al.* 1999). Infection with *Bd* may elevate HSI, as infection has been associated with congested livers (Densmore and Green 2007).

The splenosomatic index (SSI), which is the ratio of spleen weight to body weight, is a useful measure because it can reflect adverse effects of contaminants while simultaneously providing crude information on the immune status of organisms. The spleen serves as a site for the maturation of macrophages and for their interaction with T and B cells (Repetto and Baliga 1996). The SSI of fish exposed to pesticides is frequently lowered (Repetto and Baliga 1996), indicating possible immunosuppression (Fernie *et al.* 2005; Baer *et al.* 2009). In contrast, when spleens are enlarged, it frequently reflects infection or disease, and a subsequent investment in an immune response (Seppänen *et al.*, 2009).

Macrophage aggregates (MA) are distinct groupings of pigment-containing, phagocytic, resident macrophage cells that, among amphibians, are found in spleen (SMA), liver (LMA) and kidney tissues (Agius and Roberts 2003). MAs have many roles, including sequestration and detoxification of endogenous and exogenous substances, and storage and recycling of products following cell membrane and erythrocyte breakdown (Wolf and Wolfe 2005). In fish, the number, size and pigmentation of MAs appear to be affected by exposure to contaminants, and therefore, they are commonly used as biomarkers of water pollution (Agius and Roberts 2003;

Thomas 2008). In amphibians, Linzey *et al.* (2003) observed increased numbers of LMAs in marine toads (*Rhinella marina*) collected at polluted sites.

MAs also have an antigen-presenting function: they may initiate and modulate immune responses to infection (Agius and Roberts 2003; Reyes and Terrazas 2007). Consequently, their role in immune function is crucial (Reyes and Terrazas 2007). Rohr *et al.* (2008b) found that in northern leopard frogs collected from polluted wetlands, atrazine was a significant negative predictor of LMA scores, and in turn, LMA scores were a significant negative predictor of larval trematode abundance: this suggested that atrazine had an immunosuppressive effect on the frogs.

Granulomas are focal accumulations of macrophages and other cells. These may form in the liver (LGA) of amphibians exposed to pollutants, or with pathogenic infection (Linzey *et al.* 2003; Froese *et al.* 2009). Linzey *et al.* (2003) observed increased numbers of LGAs that were associated with necrotic debris, in the livers of *R. marina* collected at polluted sites.

Leucocytes are part of a primary line of defense in the innate immune system of vertebrates (Davis *et al.* 2008). Leucocyte counts are often used in toxicological studies to detect immunosuppression following exposure to contaminants (Thomas 2008). Numerous studies indicate that pesticides may depress numbers of circulating lymphocytes, eosinophils and WBCs among various organisms including amphibians (Repetto and Baliga 1996; El-Gendy *et al.* 1998; Kiesecker 2002; Christin *et al.* 2003, 2004; Forson and Storfer 2006). Leucocyte counts can also indicate changes in WBC profiles that occur with pathogenic infection (Davis *et al.* 2008). Moreover, they can indicate a stress response to both chemical exposure and pathogenic infection, because

stress often alters the N/L ratio and causes a decline in numbers of eosinophils (Davis *et al.* 2008). Stress is important to monitor because chronic stress may eventually affect normal growth and reproduction patterns (Thomas 2008), with potentially adverse consequences at the population level.

Objectives

Northern leopard frogs are exposed to pesticide products throughout their range (Smith and Keinath 2007). They are also exposed to *Bd* throughout their range, although some populations have demonstrated resistance to the pathogen (Ouellet *et al.* 2005; Longcore *et al.* 2007; Woodhams *et al.* 2008; Tennessen *et al.* 2009). Given that pesticides are known to be immunosuppressive (Taylor *et al.* 1999; Kiesecker 2002; Gendron *et al.* 2003; Gilbertson *et al.* 2003; Christin *et al.* 2004; Albert *et al.* 2007; Rohr *et al.* 2008b), it is important to test whether they can increase the susceptibility of leopard frogs to *Bd.*

I tested the hypothesis that exposure to pesticide formulations containing atrazine or glyphosate at environmentally-relevant concentrations, increases the susceptibility of post-metamorphic northern leopard frogs to infection by *Batrachochytrium dendrobatidis*. To measure levels of infection by *Bd*, molecular and histological tests were performed. In addition, I assessed lethal and sublethal impacts of the exposures by measuring survival, growth and various biomarkers of animal health and immune function. The biomarkers of animal health included HSI, the densities and sizes of LMAs and SMAs, and the densities and sizes of LGAs. The biomarkers of immune function

included SSI, the proportions of circulating leucocytes (differential WBC count), the ratio of N/L, and the ratio of WBC/RBC.

Materials and Methods

Animal collection and husbandry

In August 2007, 250 recently-metamorphosed northern leopard frogs were captured near a large pond located in the Parc de la Frayère de Rivière-aux-Pins; a wildlife reserve near Boucherville, Quebec (45°38'06''N 73°26'06''W). Immediately after capture, the snout-vent length (SVL) of each individual was measured with digital calipers, and skin swabs were taken from 20 randomly selected individuals to pre-test for infection with *Bd*. In this procedure, the entire undersurface, appendages and back of each frog were swabbed three times with a sterilized cotton swab. Each swab was placed in a sterilized Eppendorf tube and sent to the Animal Health Centre, Abbotsford, BC, for analysis by PCR. Water samples taken from the pond were analyzed for 39 commonly-used pesticides including atrazine and glyphosate, at the Centre d'expertise en analyse environnementale du Québec (CEAEQ), Quebec City, QC.

Upon arrival in the laboratory, each frog was isolated in an individual 1.84L plastic container containing 50 mL of dechlorinated, UV-treated water. The water was changed daily. Each container had two platforms that permitted the frogs to exit the water. The animals were maintained on a 16h: 8h light-dark photoperiod at a room temperature range of 21 ± 1.3°C. For the first 14 days post-capture, the frogs were given a prophylactic treatment consisting of crickets injected with oxytetracycline dissolved in distilled water [50 μg/g frog body weight] to prevent outbreaks of bacterial infections, which frequently occur among captive amphibians. Following this, the frogs were fed 1 to 2 antibiotic-free crickets every 2 days. Once per week, the crickets were dusted with phosphorus-free $CaCO_3$ powder before feeding to prevent nutrient deficiencies. All

capture methods, manipulations and experimental protocols were done in accordance with the recommendations of the Canadian Council on Animal Care (CCAC 1993).

Experimental design

The experimental design is shown in Figure 1. The frogs (n = 190) were randomly divided into three groups of individually-housed animals; i.e. a control group and 2 experimental groups that each contained 62 to 64 frogs. For 21 days, one experimental group was exposed to a target concentration of 2.1 µg/L atrazine; the other to100 µg ae/L glyphosate; while the control frogs were exposed to dechlorinated water. The pesticide solutions and water were renewed daily. At 21 days after the start of the exposures, the pesticide solutions were replaced with dechlorinated water, and the control and each treated group were subsequently divided into three approximately equal subgroups consisting of 20 to 22 frogs. Frogs in one subgroup from each main group were necropsied to evaluate effects of the 21-day pesticide exposure; a second subgroup from each main group was exposed to *Bd* four times over a 33 day period; while the third subgroup was retained as a *Bd* control. At 94 days after the start of all exposures (i.e. 72 days after the initial exposure to *Bd*), all surviving frogs were necropsied in order to: 1) assess the longer term effects of the pesticide exposures on the frogs; 2) determine if prior pesticide exposure affected the susceptibility of the post-metamorphic northern leopard frogs to *Bd* infection, and; 3) assess the combined effects of exposure to pesticides and *Bd* on frog health.

Exposures to pesticides

One day prior to the start of the experiment, stock solutions of atrazine and glyphosate were prepared at 1000X the target exposure concentrations. These were stored in 2 L amber flasks that were sealed with Teflon tape, placed inside styrofoam boxes to prevent photodegredation, and stored at 4°C.

At Day 0 of the experiment, 190 frogs were weighed (mean ± SD = 4.41 ± 1.13 g), measured (mean SVL ± SD = 38.05 ± 2.98 mm) and randomly-assigned to individual, sterilized 1L Mason jars containing 50 mL of either a pesticide solution (Aatrex® Liquid 480 or Roundup® Original), or dechlorinated water (Figure 1). The location of the jars was randomly interchanged weekly to minimize effects of uneven lighting or temperature within the laboratory, and they were placed on a slant so that the frogs could enter and leave the water at will. The mouth of each jar was covered with fiberglass screening secured with the screw-on ring to prevent escape. During the 21-day pesticide exposure, the pesticide solutions were replaced daily with freshly diluted stock solution, as was the water in the control jars.

At Day 20 of the experiment, a sample of each freshly diluted stock solution was collected and analyzed to determine the actual exposure concentrations of the pesticides. At Day 21 of the experiment, following exposure of the frogs to the pesticides, water samples from 6 randomly-chosen exposure jars per pesticide group were collected and combined for similar analysis. The glyphosate samples were analyzed by AXYS Analytical Services Ltd., Sidney BC; the atrazine samples by the National Laboratory for Environmental Testing (NLET), Environment Canada, Burlington, ON. The original stock solutions were not analyzed due to budget constraints.

The analytical results of the samples containing glyphosate were lower than expected. Consequently, a replicate stock solution was made to verify the accuracy of the preparation protocol and stability of the solution during refrigeration. This time, the water was autoclaved prior to addition of the product, because glyphosate may be degraded rapidly by microbial activity (Giesy *et al.* 2000). Three new 1/1000 dilutions were made and sent to AXYS for testing; one was frozen immediately, the others were stored in the dark at 4°C and frozen after short term refrigerated storage (i.e. after 2 and 6 days respectively).

At Day 21 of the experiment, the pesticide exposures were terminated. All frogs were weighed (mean ± SD = 5.04 ± 1.09 g) and measured (mean SVL ± SD = 40.42 ± 2.95 mm). One subgroup from each pesticide-exposed group and the control group (total n = 60) was randomly selected for necropsy. These frogs were killed by immersion in a 0.8% tricaine-methanylsulfonate (MS-222) solution, necropsied and examined for gross pathology with a dissecting microscope. Their spleen and liver were removed, rinsed with distilled water, blotted dry and weighed to the nearest 0.0001 g. The remaining frogs in each treatment group were returned to their respective jars, each of which contained 50 mL of dechlorinated water, to begin the pathogen exposure phase of the experiment (Figure 1).

Exposures to *Batrachochytrium dendrobatidis*

A culture of *Bd* (Strain JEL423) isolated during an amphibian die-off event in Panama was obtained from Dr. J.E. Longcore (University of Maine, Orono, Maine, USA). Its infectivity was confirmed by the successful infection of an American bullfrog

tadpole during a pilot experiment conducted during 2007. The culture was maintained in 1% tryptone broth at 4°C and subcultured every 3 months. For subculturing, 75 mL of freshly mixed broth were poured into 150 mL Erlenmeyer flasks, autoclaved and cooled. A 7.5 mL aliquot of the old culture and 0.75 mL of streptomycin-penicillin were added to each flask using sterile techniques. The flasks were sealed and incubated at 22°C for 10 days, then stored at 4°C until needed.

In preparation for exposure, 75 mL of *Bd* culture was warmed to 22°C and 1 mL was added to each of thirty 9 cm Petri plates that contained 1% tryptone agar. The plates were air-dried, sealed with Parafilm and incubated at 22°C for 10 days. Similar plates lacking *Bd* were also prepared to be used for sham exposures of control animals.

At Day 22 of the experiment, one day following termination of the pesticide exposures, one of the two remaining subgroups from each of the main groups of control, atrazine–exposed and glyphosate–exposed frogs was randomly selected to undergo exposure to *Bd* (Figure 1). Zoospores were harvested by flooding the inoculated Petri plates with 3 mL of dechlorinated water for 30 minutes, to trigger their discharge from *Bd* sporocysts on the agar. The suspensions in all plates were pooled into a single flask. Each plate was rinsed with an additional 1 mL of water, which was also added to the suspension. An equal volume mixture of the pooled suspension plus 0.4% trypan blue was examined on a hemocytometer to measure the concentration of zoospores and confirm their mobility. A 50 µL aliquot of the zoospore suspension was then added to the water in the jar of each frog exposed. Control jars received 50 µL of distilled water decanted from the uninoculated 1% tryptone agar plates. All jars were left upright for 24 hours to force exposure of the frogs, then the liquid in each jar was replaced with clean

dechlorinated water, and the jars were returned to their tilted position. Exposure to zoospores was repeated once weekly for 33 days. The estimated concentrations of zoospores to which the frogs were exposed during weeks 1 to 4 were, respectively: 79,600 zoospores/mL; 86,400 zoospores/mL; 130,000 zoospores/mL; 67,200 zoospores/mL. During the 33-day *Bd* exposure period, all jars were checked daily for shed skin, which is symptomatic of *Bd* infection (Pessier *et al.* 1999).

At Day 94 of the experiment, each surviving frog (n = 122) was weighed (mean ± SD = 9.53 ± 1.57 g) and measured (mean SVL ± SD = 49.25 ± 2.61 mm). A blood sample was collected by cardiac puncture using a tuberculin syringe. Three blood smears were prepared for each frog: these were fixed immediately in methanol and later stained by the Wright-Giemsa method. The frogs were then killed by immersion into 0.8% MS-222 and processed as described for Day 21. In addition, the spleen and liver of each frog was fixed in 10% neutral buffered formalin and submitted to the McGill Bone Centre (McGill University, Montreal, QC) for histological processing. The long toe from the hind, left foot was severed, fixed in 70% ethanol and sent to Trent University, Peterborough, ON, to diagnose and quantify *Bd* infection by real-time PCR, as described by Boyle *et al.* (2004). Due to time constraints, only toes of 10 *Bd*-exposed and 5 *Bd*-unexposed frogs of each pesticide treatment group (including the pesticide controls) were analyzed. Hence, only the toes of 45 randomly-selected frogs were examined by PCR. Finally, the long toe from the right hind foot of each frog was fixed in 10% formalin and submitted to the McGill Bone Centre (McGill University, Montreal, QC) for histological processing, in order to diagnose both infection by *Bd* and chytridiomycosis by histological methods, as described by Olsen *et al.* 2004.

Survival, growth and biomarkers of animal health and immune function

Survival and growth were measured, in addition to various biomarkers of animal health and immune function. The biomarkers of animal health included HSI, the densities and sizes of LMAs and SMAs, and the densities and sizes of LGAs. The biomarkers of immune function included SSI, differential WBC counts, the ratio of WBC/RBC and the ratio of N/L.

Survival, growth, HSI and SSI were measured following each experimental stage (at Days 21 and 94 of the experiment). Measurement of endpoints following the pesticide exposure (at Day 21) provided insight into the state of health of frogs entering the second experimental stage and permitted evaluation of immediate effects of exposure to the pesticides. Growth was calculated both as change in SVL and as change in body mass. The HSI and SSI were calculated by dividing fresh organ weight by total body weight. All other biomarkers (LMAs, SMAs, LGAs, differential WBC counts, WBC/RBC, N/L) were measured at the end of the experiment (at Day 94).

To measure the LMAs, SMAs and LGAs, livers and spleens from 60 randomly-selected individuals (i.e. only 10 frogs per treatment group among frogs killed at Day 94 of the experiment, due to time constraints; Figure 1) were sectioned at 5 μm and stained with hematoxylin and eosin. The histological slides were examined microscopically using 100X magnification. LMAs, SMAs or LGAs were counted with the aid of a 100 mm^2 grid placed on the slide. For LMAs, five grid squares that completely fit over part of the liver section were selected at random, and the LMAs observed within them were counted. Due to the small size of the spleen, SMAs were counted in every square of the grid that

completely fit over part of the organ. LGAs were rare, so they were also counted in every square of the grid that completely fit over liver tissue. The counts of LMAs and SMAs were converted to density per mm^2 while the data on LGA counts were treated as described below. The size of each LMA, SMA or LGA counted was then measured at 200X with the aid of an ocular eyepiece. Given that the LMAs, SMAs and LGAs were relatively round to oval, the lengths of their major and minor axes were measured, and their surface areas (μm^2) were estimated using the formula $\pi * a * b$ (a = half length of major axis; b = half length of minor axis).

Due to time constraints, the differential and total WBC counts were performed on the blood smears of only 10 or 11 randomly-selected individuals per treatment group, among the frogs killed at Day 94 (total $n = 61$). This was done to assess immune function in response to the pesticide or combined exposures. The N/L ratio was also calculated from these smears as an indicator of a stress response (Davis *et al.* 2008). Areas of the slide containing a single layer of blood cells were scanned microscopically under oil immersion in a zigzag pattern until approximately 100 WBCs were observed. All RBCs and WBCs in each field were counted. WBC identifications are based on Rouf (1969).

Indicators of infection and disease

Epidermal shedding may be used by frogs to avoid or clear infection by *Bd* (Woodhams *et al.* 2007a). Therefore, the frequency with which frogs shed their skin was monitored during the 33-day *Bd* exposure period and compared between *Bd*-exposed and *Bd*-unexposed frogs.

Susceptibility to *Bd* and hence an overall view of immune function were evaluated by previously described molecular and histological techniques (Boyle *et al.* 2004; Olsen *et al.* 2004; Hyatt *et al.* 2007). Histological examinations were performed on toes collected and fixed in formalin at Day 94 of the experiment, to diagnose infection by *Bd* and/or development of chytridiomycosis among all individuals. Following decalcification, the toes were sectioned at 5 μm along the longitudinal axis and stained with hematoxylin and eosin. To diagnose infection, each cell of the *stratum corneum* and *stratum granulosum* of the epidermis surrounding each toe was examined at 400X for sporocysts. To diagnose chytridiomycosis, the epidermis was scanned at 100X to co-localize sporocysts and hyperkeratosis as described by Olsen *et al.* (2004).

To diagnose and quantify infection by *Bd* by molecular methods, molecular analyses (i.e. real-time PCR) were performed on 45 ethanol-preserved toes collected from frogs at Day 94 of the experiment (i.e. only 10 *Bd*-exposed and 5 *Bd*-unexposed frogs per pesticide treatment group, due to time constraints within the laboratory contracted to perform the analysis), following methods described in Boyle *et al.* (2004).

Statistical analyses

Analyses were performed using SAS® version 9.1 (SAS Institute Inc. 2004). Survival was compared by a Kaplan-Meier analysis among the two pesticide treatment groups and the control frogs of the first experimental stage (Days 0 to 21 of the experiment), and among the six combined exposure treatment groups of the second experimental stage (Days 21 to 94, and Days 0 to 94 of the experiment). The log-rank statistic determined whether there were any significant differences in survival.

The initial SVLs and masses of the frogs assigned to the two pesticide treatment groups and the controls were compared by one-way factorial ANOVAs, with pesticide treatment as the single factor, to ensure that they were of equal size at the start. Growth, HSI and SSI measured at Day 21 of the experiment were compared by similar ANOVAs.

Growth from the onset of *Bd* exposure until the end of the experiment (Days 21 to 94 of the experiment), and growth over the course of both exposures (Days 0 to 94 of the experiment), were compared by two-way factorial ANOVAs, with pesticide exposure treatment, *Bd* exposure treatment and their interaction as the model terms. Two-way factorial analyses were similarly used to compare HSI, SSI, the densities and sizes of LMAs and SMAs, and the sizes of LGAs measured at Day 94 of the experiment.

Shapiro-Wilks and Levene's tests were used to check the normality of the distribution of residuals and the homogeneity of variances, respectively, for all ANOVAs, and data were transformed when necessary to meet the assumptions of analysis. Bonferroni and Scheffé's tests were used to adjust p values when multiple comparisons were performed. Up to three individuals were excluded from some analyses comparing mass, SVL, HSI and SSI, due to errors during data collection; eight individuals were excluded from the analysis to compare change in mass during the pesticide exposures (Days 0 to 21 of the experiment) for the same reason. In addition, two frogs discovered to be intersex individuals (i.e. possessing both male and female gonads), upon their necropsy at Day 94, were excluded from the analyses comparing the changes in mass, and in SVL, that occurred between Days 21 to 94, and between Days 0 to 94 of the experiment. These same intersex individuals were also excluded from the analyses to compare the HSI and SSI of frogs killed at Day 94. Where the coefficient of

determination (R^2) was small, a power analysis was performed, using the GLMPOWER procedure, to estimate sample sizes that would be required to get a significant result with 90% probability.

The data set describing the density of LGAs was zero-inflated and hence did not fit a normal distribution. Consequently, the GENMOD procedure was used to detect effects of pesticide exposure, *Bd* exposure, and their interaction on this endpoint. GENMOD fits a generalized linear model with a link function to data, and the distribution can be other than normal (SAS Institute Inc. 2004). The negative binomial distribution with a log link function provided the best fit to the data set, based on the deviance/*df* ratio, which approaches 1 as goodness of fit improves. Because the number of grid squares in which liver granulomas were counted varied between individuals, this was set as an offset term in the analysis.

GENMOD was also used to compare the blood cell ratios and frequency of epidermal shedding during the 33-day period of exposure to *Bd*. Because these data sets were recorded as proportions, the binomial distribution was fitted with a log link function.

In all cases where GENMOD was used, the deviance/*df* ratio reported some degree of overdispersion in the data (i.e. deviance/*df* > 1). Thus, the data sets were analyzed twice, allowing implementation of a scaling option during the second run (i.e. "scale=deviance" statement). This specifies an overdispersed model, and adjusts the covariance matrix and likelihood function by the scale parameter, which is fixed at a value of 1.

Results

Field results

Only glyphosate and its primary degradation product AMPA were present in the natal pond at concentrations above detectable limits (0.091 μg ae/L and 1.2 μg/L, respectively). The level of glyphosate was well below the NOEC estimated for amphibians chronically exposed to the pesticide (740 μg ae/L; Giesy *et al.* 2000), and was 1,000 fold less than the nominal concentration intended for the experimental exposure. Although, the toxicity of AMPA to amphibians has not been reported (Giesy *et al.* 2000; Govindarajulu 2008), the traces detected in the field were also well below levels considered to be toxic to fish and other organisms (Giesy *et al.* 2000). The potential effects of glyphosate and AMPA on the experiment were therefore considered negligible. All of the frogs tested for *Bd* infection in the field samples did not reveal a presence of infection.

Experimental pesticide concentrations

Upon verification of the pesticide concentrations, it was revealed that the concentration of atrazine declined in the exposure jars, but that the frogs were nonetheless exposed to levels close to the nominal concentration of 2.1 μg/L. The concentration of atrazine in the test solution prepared at Day 20 was 4.28 ± 0.04 μg/L, while the mean concentration of atrazine in the pooled samples collected from the exposure jars at Day 21 was 1.70 ± 0.26 μg/L. Glyphosate decayed rapidly during storage such that the frogs were exposed to less than the target concentration of 100 μg ae/L. The concentration measured in the sample of test solution prepared at Day 20 was 3.83 ± 0.95

μg ae/L, and the mean concentration in the pooled samples collected from the exposure jars at Day 21 was 8.13 ± 1.27 μg ae/L. The protocol for preparing the original stock solution had been adequate, because the concentration of glyphosate in the replicate stock solution prepared fresh and immediately frozen was 94.65 ± 2.90 μg ae/L. The original stock solution likely broke down within 2 days of refrigerated storage, because the concentrations in dilutions of the replicate solution stored for 2 or 6 days at 4°C were 7.00 and 7.29 μg ae/L, respectively.

Baseline data

Prior to exposure at Day 0, there was no significant difference in the SVL or body mass of the frogs randomly assigned to the three treatment groups (ANOVA for SVL: n = 189; $F_{2,186}$= 0.28, p = 0.76; ANOVA for mass: n = 188; $F_{2,185}$ = 0.73, p = 0.49).

Survival, growth and biomarkers of animal health and immune function

Survival in all treatment groups (i.e. 3 pesticide groups; 6 pesticide + *Bd* groups) ranged from 90 to 100% (i.e. mortality of 0 to 2 individuals / group) (Table 1). There was no significant difference in survival during the pesticide exposure, during exposure to *Bd*, or over the course of both exposures (Kaplan-Meier test, log-rank statistic: pesticide exposure x^2 = 2.00, df = 2, p = 0.37; *Bd* exposure x^2 = 3.79, df = 5, p = 0.58; overall x^2 = 2.66, df = 5, p = 0.75).

There was a significant difference in the change in SVL among frogs during the 21-day exposure to the pesticides (ANOVA: n = 184; $F_{2,181}$ = 4.63, p = 0.01; R^2 = 0.049) (Table 2, Figure 2). The change in SVL of frogs exposed to glyphosate was significantly

less than that of control frogs (Scheffé's test: $p = 0.02$); there was no significant difference between atrazine-exposed and control frogs ($p = 0.84$). There was no significant effect of pesticides, *Bd*, or their interaction on SVL during the second phase of the study (*Bd* exposure) (ANOVA: $n = 120$; pesticide $F_{2,114} = 0.11, p = 0.89$; *Bd* $F_{1,114} = 0.22, p = 0.64$; pesticide*Bd* $F_{2,114} = 0.36, p = 0.70$; $R^2 = 0.010$), nor was there an effect of pesticides, *Bd* or their interaction on SVL over the course of both exposures (ANOVA: $n = 120$; pesticide $F_{2,114} = 0.29, p = 0.75$; *Bd* $F_{1,114} = 0.16, p = 0.69$; pesticide*Bd* $F_{2,114} = 0.13, p = 0.88$; $R^2 = 0.008$).

Neither atrazine nor glyphosate had an effect on weight gain during the 21-day pesticide exposure period (ANOVA: $n = 179$; $F_{2,176} = 1.21, p = 0.30$; $R^2 = 0.014$) (Table 2). Similarly, the pesticides and *Bd* had no significant main or interaction effect on weight gain during subsequent exposure to *Bd* (Day 21 to 94) (ANOVA: $n = 119$; pesticide $F_{2,113} = 1.55, p = 0.22$; *Bd* $F_{1,113} = 0.38, p = 0.54$; pesticide*Bd* $F_{2,113} = 2.40, p = 0.10$; $R^2 = 0.068$). However, over the course of the entire experiment (Days 0 to 94), pesticide exposure had a significant effect on weight gain (ANOVA: $n = 121$; pesticide $F_{2,115} = 3.91, p = 0.02$; *Bd* $F_{1,115} = 0.02, p = 0.90$; pesticide*Bd* $F_{2,115} = 0.25, p = 0.78$; $R^2 = 0.068$). When pooled across the *Bd* exposure treatments, frogs exposed to atrazine gained significantly less mass than pesticide control frogs over the course of the entire experiment (Scheffé's test: $p = 0.0256$) (Figure 3). Frogs exposed to glyphosate also gained less weight than pesticide control frogs, but the difference was not significant (Scheffé's test: $p = 0.21$) (Figure 3).

No differences were found in HSI or SSI of frogs following exposure to the pesticides, at Day 21 (HSI ANOVA: $n = 59$; $F_{2,56} = 1.82, p = 0.17$; $R^2 = 0.061$) (SSI

ANOVA: $n = 59$; $F_{2,56} = 2.11, p = 0.13$; $R^2 = 0.070$) (Table 3). Following subsequent exposure to *Bd*, there were no significant main or interaction effects of the pesticide exposure or of exposure to *Bd* on HSI or SSI (HSI ANOVA: $n = 121$; pesticide $F_{2,115} = 1.88, p = 0.16$; *Bd* $F_{1,115} = 0.67, p = 0.42$; pesticide**Bd* $F_{2,115} = 0.37, p = 0.69$; $R^2 = 0.040$) (SSI ANOVA: $n = 121$; pesticide $F_{2,115} = 0.78, p = 0.46$; *Bd* $F_{1,115} = 0.20, p = 0.66$; pesticide**Bd* $F_{2,115} = 0.03, p = 0.97$; $R^2 = 0.015$).

Exposure to a pesticide, *Bd* separately, or in combination had no significant effect on the number of hepatic melanomacrophage aggregates (LMA) per mm^2 at Day 94 of the experiment (ANOVA: $n = 60$; pesticide $F_{2,54} = 2.48, p = 0.09$; *Bd* $F_{1,54} = 0.21, p = 0.65$; pesticide**Bd* $F_{2,54} = 0.91, p = 0.41$; $R^2 = 0.115$) (Table 4). Similarly, the exposures had no effect on the size of LMAs (ANOVA: $n = 60$; pesticide $F_{2,54} = 0.33, p = 0.72$; *Bd* $F_{1,54} = 0.63, p = 0.43$; pesticide**Bd* $F_{2,54} = 0.00, p = 0.99$; $R^2 = 0.023$) (Table 4).

Many of the frogs examined had no hepatic granulomas (LGA) at Day 94 of the experiment. One glyphosate-exposed-*Bd*-exposed frog had a LGA density (4.09 per mm^2) that was an extreme outlier compared to the mean (± SD) of all other frogs ($n = 59$; 0.11 ± 0.15). When included in the analysis, the result showed a significant effect of pesticide exposure on the density of LGAs (Negative Binomial Regression: $n = 60$; deviance/df = 1.16; pesticide $x^2_{2,54} = 9.89, p = 0.01$; *Bd* $x^2_{2,54} = 0.44, p = 0.51$; pesticide**Bd* $x^2_{2,54} = 2.19, p = 0.33$). *A priori* contrasts revealed that glyphosate-exposed frogs had significantly more LGAs than controls ($x^2_{1,54} = 7.25, p = 0.01$). However, based on a studentized residual of 24.33 and the assumption that the probability that other frogs sampled from the same population should exhibit such a high density of LGAs is small, the outlying datum was excluded from the analysis. Hence, exposure to a pesticide, *Bd* separately, or in

combination had no significant effect on the number of LGAs per mm^2 (Negative Binomial Regression: $n = 59$; deviance/$df = 1.28$; pesticide $x^2_{2,53} = 2.03$, $p = 0.36$; *Bd* $x^2_{2,53} = 0.65$, $p = 0.42$; pesticide**Bd* $x^2_{2,53} = 1.28$, $p = 0.53$) (Table 5). Among frogs that had LGAs, exposure to a pesticide, *Bd* separately, or in combination had no significant effect on their mean size (ANOVA: $n = 41$; pesticide $F_{2,35} = 0.89$, $p = 0.42$; *Bd* $F_{1,54} = 0.02$, $p = 0.89$; pesticide**Bd* $F_{2,54} = 0.07$, $p = 0.93$; $R^2 = 0.052$) (Table 5).

There were no significant main or interaction effects of exposure to the pesticides or *Bd* on the number and size of SMAs at Day 94 of the experiment. (SMA density ANOVA: $n = 60$; pesticide $F_{2,54} = 2.56$, $p = 0.09$; *Bd* $F_{1,54} = 2.27$, $p = 0.14$; pesticide**Bd* $F_{2,54} = 0.41$, $p = 0.66$; $R^2 = 0.132$) (SMA mean size ANOVA: $n = 60$; pesticide $F_{2,54} = 1.31$, $p = 0.28$; *Bd* $F_{1,54} = 0.67$, $p = 0.42$; pesticide**Bd* $F_{2,54} = 0.37$, $p = 0.70$; $R^2 = 0.069$) (Table 6).

The coefficients of determination (R^2) demonstrated that most models explained little of the variability in the data sets describing growth, HSI, SSI, the densities and sizes of LMAs and SMAs, and the sizes of LGAs. The GLMPOWER procedure revealed that much larger sample sizes would have been required to detect significant effects of the experimental exposures on these variables with 90% probability.

Pesticide exposure almost always, and *Bd* exposure at times, significantly affected the blood cell ratios (Tables 7, 8). However, the deviance/*df* ratios indicated minor to high overdispersion among all data sets (Tables 7, 8). After implementation of the scaling option in GENMOD, none of the experimental factors had a significant effect on hematological values (Tables 7, 8).

Indicators of infection and disease

The frogs' rate of epidermal shedding during the *Bd* exposure period was not significantly affected by either their earlier exposure to the pesticides, or by their exposure to the pesticides and *Bd* in combination. However, shedding frequency was significantly affected by *Bd* exposure alone (Logistic regression: pesticide $x^2_{2,117} = 2.01, p = 0.37$; *Bd* $x^2_{1,117} = 33.00, p < 0.0001$; pesticide**Bd* $x^2_{2,117} = 0.86, p = 0.65$). *Bd*-exposed frogs shed an average of 1.03 ± 1.03 times over 33 days, while those not exposed to *Bd* shed an average of 0.19 ± 0.54 times during that same period (Figure 4a). Most of the frogs that failed to shed skin had not been exposed to *Bd*, whereas most of the frogs that shed their skin had been exposed to the fungus (Figure 4b).

None of the toe samples examined histologically showed evidence of sporocysts or hyperkeratosis, which are indicative of *Bd* infection or of chytridiomycosis when they are observed simultaneously. However, real-time PCR analysis of one toe sample from a frog exposed to both atrazine and *Bd* tested positive for *Bd* infection, although with a low DNA copy number (= 1.6).

Discussion

This study suggested that each of the pesticide formulations reduced the growth of post-metamorphic northern leopard frogs, even at low exposure concentrations. No other effects of the pesticides were detected on animal health or immune function when comparing survival, HSI, SSI, MAs, LGAs and leucocyte responses. No effects of *Bd* could be detected on any of the biomarkers when exposure to *Bd* occurred independently or following exposure to the pesticides. Overall, the frogs appeared to display resistance to *Bd*, and prior exposure to low levels of two pesticides did not appear to diminish this resistance.

Experimental pesticide concentrations

Atrazine-exposed frogs were exposed to levels close to the intended concentration of 2.1 μg/L for 21 days. Glyphosate-exposed frogs were likely exposed to concentrations of approximately 95 μg ae/L for the first 2 days, then to approximately 6 μg ae/L over the next 19 days. Although this was well below the intended concentration of 100 μg ae/L, glyphosate reportedly dissipates rapidly from natural aquatic environments as it binds to particles and sediment and is broken down by microbial activity (Giesy *et al.* 2000). For instance, in a watershed of British Columbia, a glyphosate residue of 162 μg/L measured in stream water following an application of Roundup® fell to <1 μg/L within 96 h post-application, as it likely adhered to bottom sediments and became biologically unavailable (Feng *et al.* 1990). Hence, both of the pesticide exposure treatments were environmentally relevant, despite the rapid degradation of glyphosate.

Effects of the pesticides

Survival

The concentration of atrazine used in this study did not directly affect survival. Two previous studies reported similar results at concentrations up to and surpassing environmental levels (Christin *et al.* 2004; Allran and Karasov 2001). In one case, metamorphosed leopard frogs were exposed to 2.1, 21 or 210 µg/L of atrazine contained in a mixture of chemicals for 21 days (Christin *et al.* 2004); in the other, they were exposed to between 1,560 and 20,000 µg/L of atrazine for 14 days (Allran and Karasov 2001). Therefore, it is unlikely that the survival of post-metamorphic leopard frogs is threatened by atrazine concentrations reported in North American surface waters through direct toxicity (Allran and Karasov 2001).

Several studies have examined the effects of glyphosate and its formulations on pre-metamorphic stages of northern leopard frogs (Chen *et al.* 2004; Edginton *et al.* 2004b; Howe *et al.* 2004; Relyea 2004, 2005, 2009; Thompson *et al.* 2004; Wojtaszek *et al.* 2004; Trumbo 2005; Relyea and Jones 2009). In contrast, although some studies have investigated similar effects on post-metamorphic stages of other frog species (Relyea, 2005; Dinehart *et al.* 2009), there has been no work on post-metamorphic stages of northern leopard frogs in particular. In this study, the glyphosate formulation did not affect survival of the leopard frogs. As the NOEC estimated for amphibians chronically exposed to glyphosate is 740 µg ae/L (Giesy *et al.* 2000), this is not surprising. However, the presence of POEA makes the toxicity of various glyphosate formulations difficult to predict because studies rarely express exposure concentrations in terms of POEA concentration. POEA has shown the capacity to significantly increase the toxicity of

glyphosate formulations among exposed amphibians (Mann *et al.* 2009). For this reason, future studies exposing amphibians to POEA-containing glyphosate formulations should consider measuring and reporting the concentration of both glyphosate and POEA.

Growth

The atrazine formulation significantly reduced gain in mass over the 94-day experiment. Previous studies have demonstrated that atrazine reduces growth in larval amphibians (Langlois *et al.* 2010; Rohr and McCoy 2010) and fish (del Carmen Alvarez and Fuiman 2005; McCarthy and Fuiman 2008; Rohr and McCoy 2010). Given that atrazine affects the timing of metamorphosis, elevates locomotor activity, reduces antipredator behaviour, induces gonadal abnormalities and reduces growth rates in amphibians, there is a growing consensus that it is a potent endocrine disruptor in this group (Hayes *et al.* 2010b, Rohr and McCoy 2010), which could produce the result observed here.

Although the weight gain of atrazine-exposed frogs was reduced, the gain in SVL was unaffected. In fish, this response, which reflects a lowered condition factor, is normally interpreted as evidence of a decline in body fat or glycogen stores, possibly due to a raised metabolic rate, reduced energy intake, or enhanced rate of fat metabolism (Schlenk *et al.* 2008). Evidence that atrazine can accelerate metabolism in amphibians comes from a previous exposure study where larval African clawed frogs exposed to 400 μg/L atrazine exhibited reduced fat bodies (i.e. lipid reserves attached to the gonads) and an increased expression of genes involved in proteolysis, digestion and carbohydrate metabolism, compared to controls (Langerveld *et al.* 2009).

Alternatively, a lower condition factor in frogs could be explained by dehydration following disruption of behavioural or physiological mechanisms that regulate water balance. Both types of mechanisms are regulated by a number of hormones, including arginine vasotocin, sex hormones, hydrins, angiotensin II, prolactin and adrenaline (Wells 2007). Based on the evidence that atrazine upsets endocrine functions in amphibians (Hayes *et al.* 2010a, Rohr and McCoy 2010), it seems reasonable that the pesticide could affect these systems. In fact, post-metamorphic streamside salamanders (*Ambystoma barbouri*) exposed to 40 or 400 µg/L atrazine during larval development exhibited lower body mass, increased activity, fewer water-conserving behaviors (e.g. huddling, covering exposed skin) and accelerated water loss, compared to controls, at 4 and 8 months following exposure (Rohr and Palmer 2005). It was proposed that neuroendocrine functions governing water-conserving behaviours in the salamanders were disturbed over the long-term (Rohr and Palmer 2005). In the current study, atrazine-exposed frogs could have become dehydrated if they increased their activity or spent less time in water, however, these behaviours were not monitored. In retrospect, monitoring hormone levels, activity levels, hematocrit, and/or water-conserving behaviours could have helped to elucidate a mode of action for the effects observed on mass gain (Rohr and Palmer 2005; Wells 2007; Rosenblum *et al.* 2009). Importantly, if atrazine has a long-term effect on normal osmoregulatory function, exposed amphibians face a substantial risk of mortality because they are particularly susceptible to desiccation (Wells 2007; Rohr and McCoy 2010).

The glyphosate formulation significantly reduced gain in SVL during the 21-day pesticide exposure. This effect has been previously reported in larval amphibians,

perhaps because POEA also disrupts normal endocrine functions (Howe *et al.* 2004). Howe *et al.* (2004) coincidentally measured smaller SVLs with other developmental effects (damaged and shortened tails, abnormal gonads, reduced time to metamorphosis) among larval leopard frogs exposed to pure POEA and to two POEA-containing herbicides (Roundup® Original, Roundup® Transorb), but not among tadpoles exposed to technical grade glyphosate. Cauble and Wagner (2005) reported reduced SVL at metamorphosis in cascades frogs (*Rana cascadae*) exposed to a Roundup® formulation during larval development, while Relyea (2009) observed no effect on mass at metamorphosis (SVL was not measured) among larval leopard frogs and gray tree frogs (*Hyla versicolor*) exposed to technical grade glyphosate.

Interestingly, when measured between Days 21 and 94, or between Days 0 and 94 of the experiment, growth of glyphosate-exposed frogs did not differ from controls. This suggests that the effects on growth ceased upon termination of exposure to the pesticide. Hence, reduced growth may have been a secondary effect of stress activating the adrenergic system and the hypothalamic-pituitary-interrenal axis (Thomas 2008). Alternatively, energy for growth may have been redirected toward detoxification processes (Mann *et al.* 2009).

While the reduction in growth among glyphosate-exposed frogs was significant, it differed by less than 1mm from that of control frogs. Whether this size difference would have severe repercussions on the survival of adult leopard frogs is questionable, but warrants investigation. In agricultural settings, POEA-containing Roundup® formulations are frequently applied prior to, and several times after crop emergence (Giesy *et al.* 2000), resulting in surface water contamination that occurs in pulses. If POEA is the

component responsible for altered growth (Howe *et al.*, 2004), its half-life may be up to 20 times longer than that of glyphosate (i.e. 14 to 41 days for POEA compared to 2 to 14 days for glyphosate) (Giesy *et al.* 2000; but see Wang *et al.* 2005), and it may persist between applications. Hence, aqueous exposures to POEA could potentially last much longer than 21 days and cause more significant growth reductions in frogs than were observed in this study.

Biomarkers of animal health

Lesions and other histopathological changes in the liver are sensitive biomarkers of pollution in fish (Hinton *et al.*, 2008). Liver damage can disrupt metabolic homeostasis and the function of vital biological processes (Hinton *et al.*, 2008). Thus, the fact that the HSIs, LMAs and LGAs were all unaltered by exposure to the pesticides suggests that the chemicals were not hepatotoxic. However, the exclusion of an extreme outlier from the data describing the density of LGAs eliminated a significant finding that exposure to the glyphosate formulation caused the formation of significantly more hepatic granulomas. Interestingly, the three frogs with the highest densities of hepatic LGAs among the 60 that were examined were glyphosate-exposed. Given this, the fact that exposure to the glyphosate formulation significantly reduced growth, and the knowledge that reduced growth can occur when energy is redirected toward detoxification processes in the liver (Schlenk *et al.*, 2008; Mann *et al.*, 2009), it would be worthwhile to reinvestigate the possibility that POEA-containing glyphosate formulations have a hepatotoxic effect on anurans. If conducted, the investigation should evaluate the effects of glyphosate and

POEA independently, and combined in a formulation, given the evidence that POEA may contribute most or all of the toxicity to the formulated product (Howe *et al.*, 2004).

Biomarkers of immune function

The biomarkers selected to monitor immune function showed no significant effects of the pesticides. However, they could not provide a full assessment of the amphibian immune system due to its complexity, and potential immunosuppressive effects of the pesticides cannot be ruled out. The mean SSIs of the pesticide-exposed frogs were lower than those of control frogs immediately after the chemical exposures (i.e. at Day 21 of the experiment), but there was large variation within the experimental groups, and no significant differences were found. No differences were found in the hematological data or blood cell ratios between pesticide-exposed and control frogs either, after adjustment for overdispersion.

Effects of *Batrachochytrium dendrobatidis*

Survival

Neither exposure to *Bd*, nor the combined exposures to the pesticides and *Bd*, had a significant effect on survival. The leopard frogs were exposed individually to between 3.36×10^6 and 6.50×10^6 fungal zoospores during each of four 24-hour exposure events over a 33-day period. Less than 10% mortality (i.e. ≤ 2 of 20 to 23 individuals) occurred in any group, and none of the frogs exposed only to *Bd* died within 72 days of the first exposure to *Bd*. In contrast, amphibian species that exhibit high susceptibility to *Bd* frequently die within 18 to 48 days of exposure (Woodhams *et al.* 2007b). Moreover,

mortality may occur at much lower doses than those used here (Carey *et al.* 2006; Woodhams *et al.* 2007a). Exposure of western toads (*Anaxyrus boreas*) to as few as 10,000 zoospores for 1 day resulted in 100% mortality within 42 days (Carey *et al.* 2006). Exposure of three Australian frog species (*Litoria caerulea, Litoria chloris, Mixophyes fasciolatus*) to 5 x 10^3 zoospores for 15 h was sufficient to cause 65-95% mortality within 108 days (Woodhams *et al.* 2007a).

Growth

Neither exposure to *Bd,* nor the combined exposures significantly affected frog growth. In contrast, a previous study showed that recently-metamorphosed foothill yellow-legged frogs exposed to *Bd* were half the size of control frogs at two months post-exposure. (Davidson *et al.* 2007). Foothill yellow-legged frogs and leopard frogs each appear to have effective defenses against *Bd*, because their survival was unaffected following experimental exposures (Davidson *et al.* 2007; this study). However, longer-term reduced growth could eventually result in population declines for yellow-legged frogs (Davidson *et al.* 2007), while this appears to be less of a concern for resistant populations of leopard frogs.

Biomarkers of animal health and immune function

None of the experimental treatments in this study appear to have caused undue stress to the frogs, based on the hematological data (Davis *et al.* 2008; Davis 2009). The N/L ratio of amphibians generally increases when they experience stress because neutrophil counts tend to rise while lymphocyte counts fall (Davis *et al.* 2008). An

important sign of stress is exhibited once the N/L ratio reaches ≥ 0.67 (Davis 2009). Here, the mean N/L ratio across the five treatment groups and the controls at the end of the experiment ranged between 0.03 to 0.11, and were well within the normal reference range for amphibians (< 0.01 to 0.67) (Davis 2009).

Neither exposure to *Bd,* nor the combined exposures significantly affected HSI, MAs, LGAs, SSI or the other leucocyte responses. In contrast, previously published pathology reports indicate that *Bd* infection can cause congestion of the internal organs (Densmore and Green 2007), suggesting that organosomatic indices may rise. Another earlier study observed increased numbers of neutrophils (neutrophilia) and decreased numbers of eosinophils (eosinopenia) in bullfrog tadpoles heavily infected with *Bd* (Davis *et al.* 2010). While these leucocyte trends can indicate that high levels of stress are associated with infection, neutrophilia can alternatively signal substantial host tissue damage or secondary bacterial invasions (Davis *et al.* 2010). Another study reported reduced numbers of neutrophils (neutropenia), eosinopenia and increased numbers of basophils (basophilia) in post-metamorphic Australian red-eyed tree frogs (*Litoria chloris*) infected by *Bd,* which may reflect movement of WBCs towards sites of infection (Woodhams *et al.* 2007a).

In short, the results of the present study suggest that exposure to *Bd* and the combined exposures did not have significant effects on the health and immune function of the leopard frogs. It is premature, however, to conclude that the immune system of the frogs did not respond to *Bd* exposure, because unmonitored components of the ranid immune system such as AMPs could have been involved in an immune response to the fungus (Woodhams *et al.* 2007b; Tennessen *et al.* 2009).

In addition, it should be emphasized that the low coefficients of determination (R^2) associated with the ANOVAs, the overdispersion in data sets analyzed by GENMOD, and the results of the power analyses suggested that uncontrolled factors unaccounted for in the statistical models influenced the individual responses of the frogs to both the pesticides and *Bd*. These factors likely include genetic background, level of sexual maturity, sex, and natural exposure to previous stressors (Hinton *et al.*, 2008; Schlenk *et al.*, 2008). First, because the experimental animals were collected in nature as froglets and not from a single egg mass, the genetic heritage of the frogs likely differed considerably, and incidentally increased the range of responses to the experimental stressors. Second, upon necropsy at Day 94 of the experiment, most frogs were approaching sexual maturity, as evidenced by the development of nuptial thumb pads on many males and enlarged eggs inside many females. Sexual development demands large amounts of energy, and trade-off theory predicts that each individual has a limited amount of energy that must be allocated toward all physiological processes (McCallum and Trauth 2007). Consequently, depending on the individual degree of progression toward sexual maturity, more or less energy may have been devoted toward other processes such as immune defense and growth. Third, the inclusion of sex as a factor in the statistical models was possible, but this created a severely unbalanced experimental design that was difficult to analyze and interpret. A study performed uniquely on males or females, or with an equal number of each sex in each data cell would have been ideal. Finally, the post-metamorphic life stage of the frogs upon collection meant that they had already been exposed to various abiotic and biotic factors, including parasites and other antigen-stimulants, which would have shaped their physiology and immune systems in

different ways. In short, controlling for some or all of these factors, and/or increasing the overall sample size would have helped to better ascertain the impact of the experimental stressors on health and immune function.

Indicators of infection and disease

Histological examination of toe sections from 63 *Bd*-exposed frogs produced no positive results for infection or for the disease chytridiomycosis, and the molecular tests conducted on the toes of 30 *Bd*-exposed frogs only produced a single diagnosis of light *Bd* infection. Although a bullfrog tadpole was successfully infected with the fungal culture in a pilot experiment, and although the zoospores were observed to be active prior to each *Bd* exposure event during the present experiment, failure to infect the leopard frogs could be due to attenuation of the culture or an error in experimental technique. However, previous studies have provided strong evidence that some northern leopard frogs are resistant to *Bd* (Ouellet *et al.* 2005; Longcore *et al.* 2007; Woodhams *et al.* 2008; Tennessen *et al.* 2009). Moreover, during the exposures, *Bd*-exposed leopard frogs shed their skin significantly more frequently than *Bd*-unexposed frogs: an increased frequency of skin shedding (ecdysis) is a known symptom of infection, and may be used by frogs to resist or clear *Bd* infections (Woodhams *et al.* 2007a). These observations lend support to the possibility that the leopard frogs used in the present study displayed resistance to the fungus.

Conclusions

Neither the atrazine, nor the glyphosate formulation caused direct mortality of the frogs at low and environmentally relevant concentrations. However, even at these low levels, each pesticide had a sublethal effect. The glyphosate formulation temporarily suppressed growth, perhaps due to the presence of POEA. The atrazine formulation had a longer-lasting suppressive effect on mass gain. Reduced growth could translate into lowered fitness for individuals and contribute to amphibian population declines.

In addition, the post-metamorphic northern leopard frogs from the sampled population did not appear to be susceptible to *Bd*, and prior exposure to environmentally-relevant concentrations of the pesticide formulations did not alter this trait. The *Bd*-exposed frogs did not display clinical symptoms of chytridiomycosis or suffer increased mortality, whether they were pre-exposed to the pesticides or not. Their defense mechanism against *Bd* was apparently cost efficient, because *Bd*-exposure did not affect growth. Given that the *Bd*-exposed frogs shed their skin excessively, ecdysis may have helped to prevent or eliminate infection. Future studies should examine the combined effects of these pesticides and *Bd* on susceptible populations of leopard frogs and on other susceptible anurans.

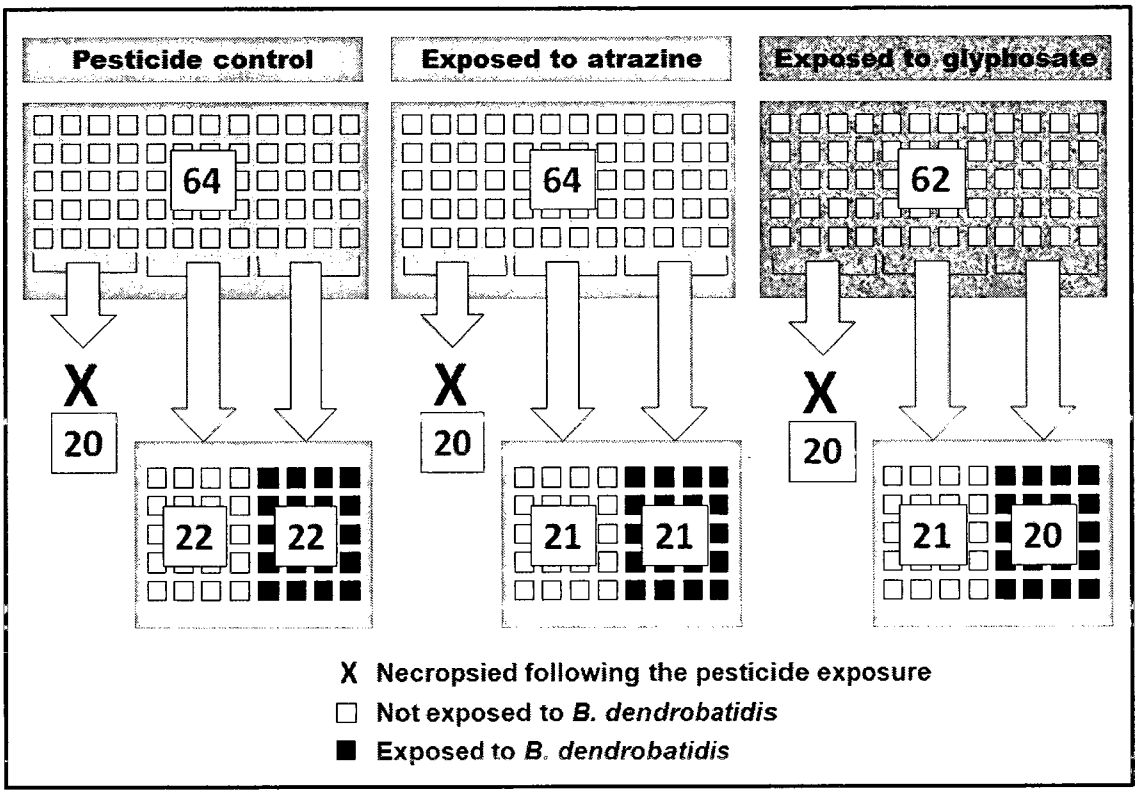

Figure 1. Schematic diagram of the experimental design. At Day 0, 62-64 northern leopard frogs (*Lithobates pipiens*) were placed into one of three treatment groups (atrazine, glyphosate or control). Frogs in the pesticide groups were exposed for 21 days while control frogs were kept in dechlorinated water. Following this, 20 frogs from the control and each treatment group were necropsied to obtain data on the effects of chronic exposure to the pesticide. Beginning at Day 22 of the experiment, all surviving frogs were kept in dechlorinated water. Half of the frogs in each pesticide treatment group were given 4 weekly exposures to the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), while the other half received sham exposures, thereby creating 6 treatment groups. At Day 94 of the experiment, the survivors were killed to assess the combined effects of exposure to the pesticide and *Bd*.

Table 1. Kaplan-Meier analysis of survival of northern leopard frogs (*Lithobates pipiens*) following exposure to **(a)** pesticides, **(b)** *Batrachochytrium dendrobatidis* and **(c)** both exposures.

Treatment groups*	$n_{initial}$	n_{final}	% Survival	*df*	x^2	*p*
(a) *following exposure to the pesticides (Days 0-21)*						
A	64	62	96.88	2	2.0006	0.3678
G	62	61	98.39			
C	64	64	100			
(b) *following exposure to Bd (Days 21-94)*						
A	21	21	100	5	3.7937	0.5795
A + *Bd*	21	20	95.24			
G	21	20	95.24			
G + *Bd*	20	18	90			
C	22	21	95.45			
C + *Bd*	22	22	100			
(c) *following both exposures (Days 0-94)*						
A	23	21	91.30	5	2.6608	0.7521
A + *Bd*	21	20	95.24			
G	22	20	90.91			
G + *Bd*	20	18	90			
C	22	21	95.45			
C + *Bd*	22	22	100			

*Frogs exposed to atrazine (A), glyphosate (G), atrazine + *Batrachochytrium dendrobatidis* (A + *Bd*), glyphosate + *Bd* (G + *Bd*), controls (C), and pesticide controls + *Bd* (C + *Bd*).

Table 2. Mean (± SD) changes in body mass and snout-vent length (SVL) of northern leopard frogs (*Lithobates pipiens*) following exposure to **(a)** pesticides, **(b)** *Batrachochytrium dendrobatidis* and **(c)** both exposures.

Treatment groups*	*n*	Change in SVL (mm)†	*n*	Change in Body Mass (g)
(a) *following exposure to the pesticides (Days 0-21)*				
A	61	2.68 ± 1.36 ^{ab}	60	0.92 ± 0.47
G	59	2.12 ± 1.44 ^{b}	57	0.95 ± 0.46
C	64	2.83 ± 1.26 ^{a}	62	1.04 ± 0.38
(b) *following exposure to Bd (Days 21-94)*				
A	21	8.50 ± 2.70	21	3.86 ± 0.83
A + *Bd*	20	8.81 ± 1.56	20	4.25 ± 0.66
G	19	8.63 ± 1.81	19	4.38 ± 0.91
G + *Bd*	18	8.38 ± 2.37	18	3.95 ± 0.98
C	20	8.14 ± 2.33	20	4.43 ± 0.94
C + *Bd*	22	8.92 ± 1.34	21	4.29 ± 0.65
(c) *following both exposures (Days 0-94)* §				
A	21	10.92 ± 3.10	21	4.80 ± 0.94
A + *Bd*	19	11.35 ± 1.76	20	5.02 ± 0.63
G	19	10.85 ± 2.93	20	5.12 ± 1.08
G + *Bd*	18	10.42 ± 3.04	18	4.99 ± 1.10
C	20	11.26 ± 2.73	20	5.39 ± 1.09
C + *Bd*	23	10.93 ± 2.76	22	5.47 ± 0.82

*Frogs exposed to atrazine (A), glyphosate (G), atrazine + *Batrachochytrium dendrobatidis* (A + *Bd*), glyphosate + *Bd* (G + *Bd*), controls (C), and pesticide controls + *Bd* (C + *Bd*). †Different superscripts (a, b) identify significantly different mean SVLs (one-way ANOVA, Scheffé's test, $p < 0.05$). § See text for details.

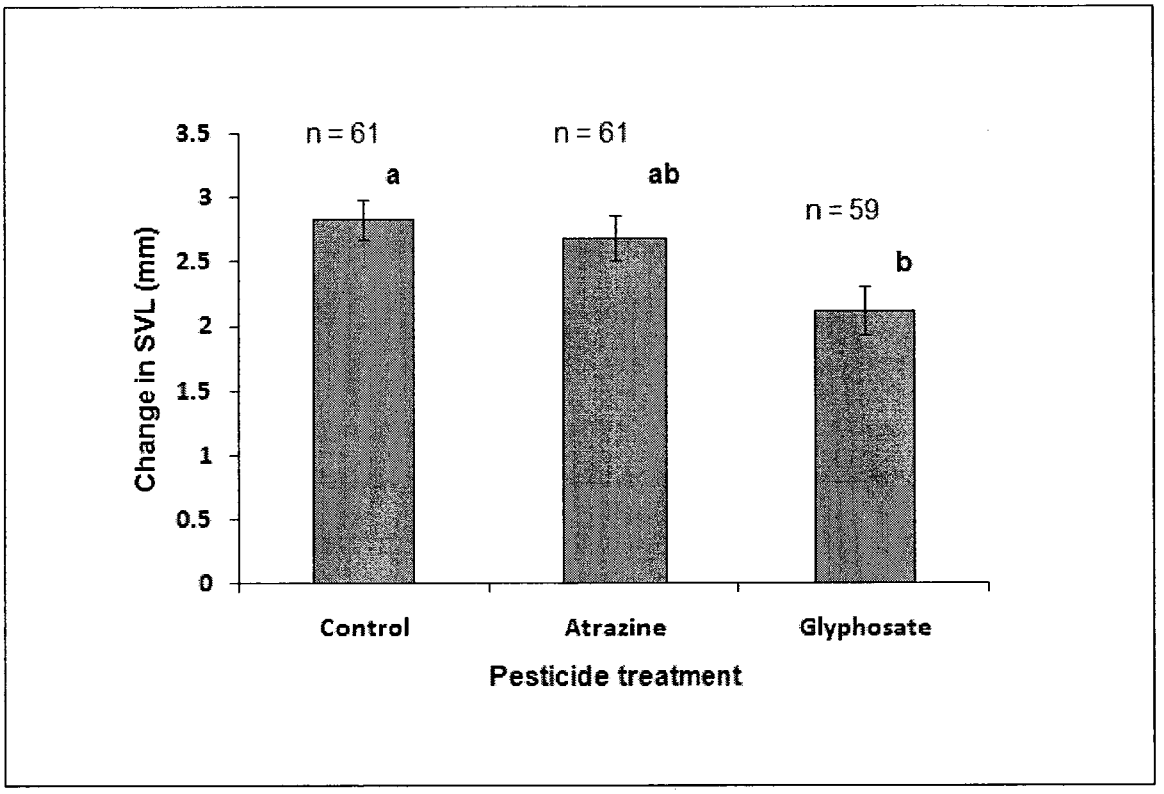

Figure 2. Mean (± SE) gain in snout-vent length (SVL) of northern leopard frogs (*Lithobates pipiens*) exposed to pesticides. Superscripts indicate significant differences among the treatment groups (one-way ANOVA, Scheffé's test, $p < 0.05$).

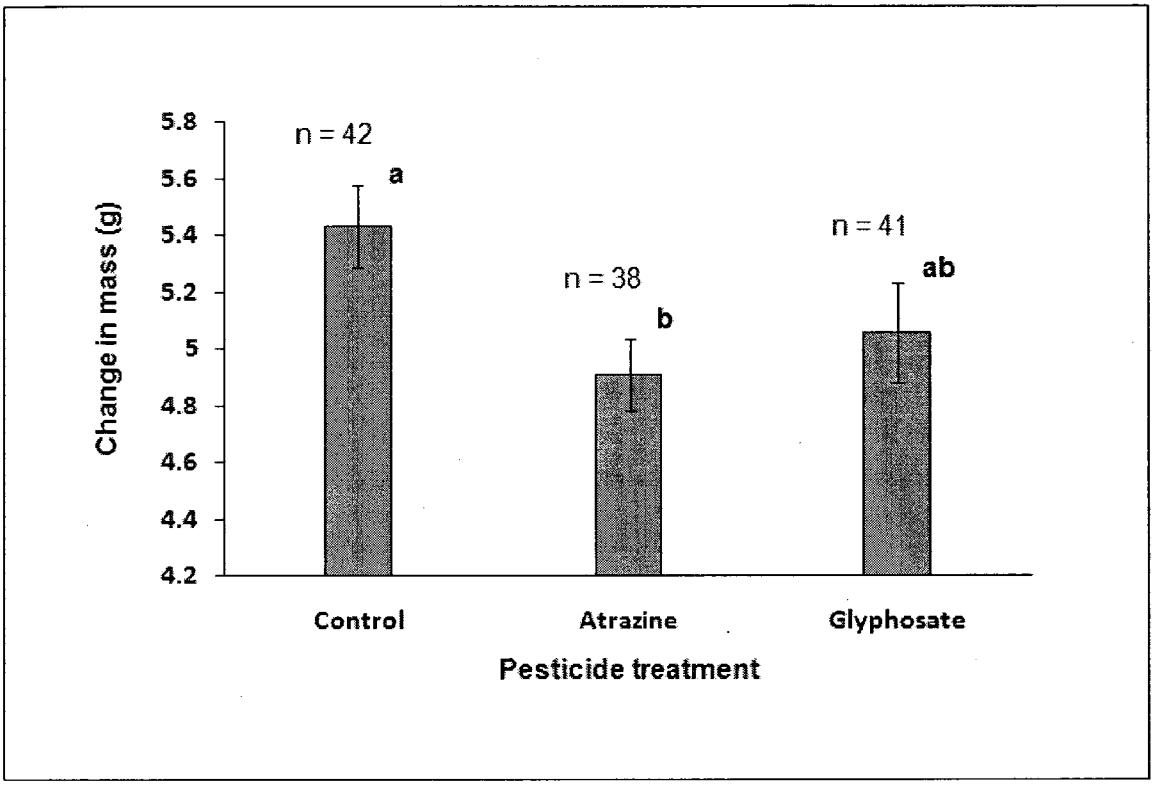

Figure 3. Mean (± SE) gain in body mass of northern leopard frogs (*Lithobates pipiens*) exposed to pesticides and *Batrachochytrium dendrobatidis*. Superscripts indicate significant differences among treatment groups. Frogs are pooled across *Bd* exposure treatments.

Table 3. Mean (± SD) hepatosomatic (HSI) and splenosomatic (SSI) indices of northern leopard frogs (*Lithobates pipiens*) necropsied following exposure to **(a)** pesticides and **(b)** pesticides and *Batrachochytrium dendrobatidis*.

Treatment groups*	*n*	HSI	SSI
(a) *following exposure to the pesticides (Days 0-21)*			
A	19	0.0315 ± 0.0072	0.0039 ± 0.0018
G	19	0.0344 ± 0.0071	0.0037 ± 0.0023
C	21	0.0308 ± 0.0043	0.0055 ± 0.0041
(b) *following both exposures (Days 0-94)*			
A	21	0.0255 ± 0.0068	0.0031 ± 0.0016
A + *Bd*	20	0.0247 ± 0.0038	0.0033 ± 0.0021
G	20	0.0251 ± 0.0056	0.0033 ± 0.0016
G + *Bd*	17	0.0256 ± 0.0042	0.0037 ± 0.0017
C	20	0.0227 ± 0.0041	0.0032 ± 0.0025
C + *Bd*	23	0.0251 ± 0.0072	0.0033 ± 0.0029

*Frogs exposed to atrazine (A), glyphosate (G), atrazine + *Batrachochytrium dendrobatidis* (A + *Bd*), glyphosate + *Bd* (G + *Bd*), controls (C), and pesticide controls + *Bd* (C + *Bd*).

Table 4. Mean (± SD) density and size of melanomacrophage aggregates in the liver (LMA) of northern leopard frogs (*Lithobates pipiens*) exposed to pesticides and pesticides plus *Batrachochytrium dendrobatidis*.

Treatment groups*	*n*	LMA density (/ mm^2)	LMA size (μm^2)
A	10	7.76 ± 3.31	20.99 ± 7.64
A + *Bd*	10	8.48 ± 2.36	19.42 ± 5.33
G	10	5.94 ± 2.29	25.21 ± 14.58
G + *Bd*	10	7.06 ± 2.12	21.49 ± 7.81
C	10	7.10 ± 2.71	22.91 ± 12.35
C + *Bd*	10	6.16 ± 2.37	20.22 ± 6.49

*Frogs exposed to atrazine (A), glyphosate (G), atrazine + *Batrachochytrium dendrobatidis* (A + *Bd*), glyphosate + *Bd* (G + *Bd*), controls (C), and pesticide controls + *Bd* (C + *Bd*).

Table 5. Mean (± SD) density and size of granulomas in the liver (LGA) of northern leopard frogs (*Lithobates pipiens*) exposed to pesticides and pesticides plus *Batrachochytrium dendrobatidis.*

Treatment groups*	*n*	LGA density (/ mm^2)	*n*	LGA size (μm^2)
A	10	0.13 ± 0.13	9	67.46 ± 43.92
A + *Bd*	10	0.07 ± 0.06	7	61.29 ± 34.87
G	10	0.19 ± 0.29	8	111.74 ± 95.35
G + *Bd*	9	0.10 ± 0.15†	5	111.25 ± 101.75
C	10	0.07 ± 0.08	7	79.68 ± 43.59
C + *Bd*	10	0.10 ± 0.10	5	97.60 ± 79.01

*Frogs exposed to atrazine (A), glyphosate (G), atrazine + *Batrachochytrium dendrobatidis* (A + *Bd*), glyphosate + *Bd* (G + *Bd*), controls (C), and pesticide controls + *Bd* (C + *Bd*). † One extreme outlier excluded from analysis.

Table 6. Mean (± SD) density and size of melanomacrophage aggregates in the spleen (SMA) of northern leopard frogs (*Lithobates pipiens*) exposed to pesticides and pesticides plus *Batrachochytrium dendrobatidis.*

Treatment group*	*n*	SMA density (/ mm^2)	SMA size (μm^2)
A	10	6.62 ± 2.75	21.15 ± 8.70
A + *Bd*	10	8.60 ± 5.94	21.98 ± 9.76
G	10	5.65 ± 4.14	26.42 ± 9.69
G + *Bd*	10	7.56 ± 2.06	23.69 ± 14.29
C	10	8.96 ± 3.71	20.11 ± 5.13
C + *Bd*	10	9.41 ± 2.82	18.29 ± 3.24

*Frogs exposed to atrazine (A), glyphosate (G), atrazine + *Batrachochytrium dendrobatidis* (A + *Bd*), glyphosate + *Bd* (G + *Bd*), controls (C), and pesticide controls + *Bd* (C + *Bd*).

Table 7. Mean (± SD) leucocyte counts among northern leopard frogs (*Lithobates pipiens*) exposed to pesticides and pesticides plus *Batrachochytrium dendrobatidis*.

Leucocyte	*n*	Treatment* group	Cell count (/ 100 WBCs)	Deviance/*df*	Factor	*F*	X^2	*p*
Basophils	10	A	3.99 ± 3.83	3.38	**Pesticide**	1.59	3.17	0.2045
	10	A + *Bd*	3.75 ± 3.13		*Bd*	0.02	0.02	0.8814
	10	G	6.12 ± 3.43		Pesticide**Bd*	0.14	0.78	0.8678
	11	G + *Bd*	5.25 ± 4.14					
	10	C	3.58 ± 3.37					
	10	C + *Bd*	4.00 ± 3.04					
Neutrophils	10	A	5.05 ± 9.23	5.50	**Pesticide**	1.13	2.27	0.3216
	10	A + *Bd*	3.11 ± 3.06		***Bd***	3.47	3.47	0.0624
	10	G	7.05 ± 7.62		Pesticide**Bd*	0.12	0.23	0.8913
	11	G + *Bd*	4.86 ± 3.15					
	10	C	5.37 ± 5.39					
	10	C + *Bd*	2.60 ± 2.23					
Eosinophils	10	A	0.90 ± 0.88	2.17	**Pesticide**	2.54	5.08	0.0751
	10	A + *Bd*	1.43 ± 1.60		*Bd*	0.02	0.02	0.8859
	10	G	1.20 ± 1.48		Pesticide**Bd*	0.43	0.87	0.6489
	11	G + *Bd*	0.81 ± 0.87					
	10	C	2.12 ± 2.53					
	10	C + *Bd*	2.18 ± 2.69					

Table 7. *Continued.*

Leucocyte	*n*	Treatment* group	Cell count (/ 100 WBCs)	Deviance/*df*	Factor	*F*	X^2	*p*
Monocytes	10	A	7.08 ± 3.58	4.45	**Pesticide**	0.88	1.76	0.4151
	10	A + *Bd*	4.43 ± 4.10		*Bd*	0.18	0.18	0.6744
	10	G	8.28 ± 4.21		**Pesticide*Bd**	1.36	2.72	0.2563
	11	G + *Bd*	6.78 ± 8.55					
	10	C	4.65 ± 3.60					
	10	C + *Bd*	6.91 ± 5.86					
Lymphocytes	10	A	81.38 ± 12.94	5.29	**Pesticide**	1.36	2.71	0.2579
	10	A + *Bd*	84.20 ± 61.21		***Bd***	1.08	1.08	0.2990
	10	G	75.89 ± 10.08		Pesticide*Bd	0.31	0.62	0.7323
	11	G + *Bd*	80.47 ± 73.51					
	10	C	81.25 ± 78.27					
	10	C + *Bd*	81.40 ± 9.04					

*Frogs exposed to atrazine (A), glyphosate (G), atrazine + *Batrachochytrium dendrobatidis* (A + *Bd*), glyphosate + *Bd* (G + *Bd*), controls (C), and pesticide controls + *Bd* (C + *Bd*). Analysis performed in GENMOD (SAS). Binomial distribution fit to the data with a log link function. Deviance/df > 1 demonstrates overdispersion of data. Bolded factors showed significant effect prior to the scaling adjustment. *F* and *p* values shown were obtained after scaling, and significant effects of the experimental factors are no longer detected ($p > 0.05$).

Table 8. Mean (± SD) blood cell ratios among northern leopard frogs (*Lithobates pipiens*) exposed to pesticides and pesticides plus *Batrachochytrium dendrobatidis*.

Ratio examined	*n*	Treatment group*	Ratio counted	*Deviance/df*	Factor	*F*	X^2	*p*
Neutrophils / Lymphocytes	10	A	0.09 ± 0.19	7.13	**Pesticide**	1.09	2.19	0.3350
	10	A + *Bd*	0.03 ± 0.04		***Bd***	3.04	3.04	0.0810
	10	G	0.11 ± 0.14		Pesticide**Bd*	0.06	0.11	0.9442
	11	G + *Bd*	0.06 ± 0.04					
	10	C	0.07 ± 0.08					
	10	C + *Bd*	0.03 ± 0.03					
WBC / RBC	10	A	0.03 ± 0.01	28.57	Pesticide	0.04	0.08	0.9613
	10	A + *Bd*	0.04 ± 0.02		***Bd***	0.55	0.55	0.4600
	10	G	0.04 ± 0.02		**Pesticide*Bd**	0.83	1.66	0.4365
	11	G + *Bd*	0.06 ± 0.11					
	10	C	0.04 ± 0.02					
	10	C + *Bd*	0.03 ± 0.01					

*Frogs exposed to atrazine (A), glyphosate (G), atrazine + *Batrachochytrium dendrobatidis* (A + *Bd*), glyphosate + *Bd* (G + *Bd*), controls (C), and pesticide controls + *Bd* (C + *Bd*). Analysis performed in GENMOD (SAS). Binomial distribution fit to the data with a log link function. Deviance/df > 1 demonstrates overdispersion of data. Bolded factors showed significant effect prior to the scaling adjustment. *F* and *p* values shown were obtained after scaling, and significant effects of the experimental factors are no longer detected ($p > 0.05$).

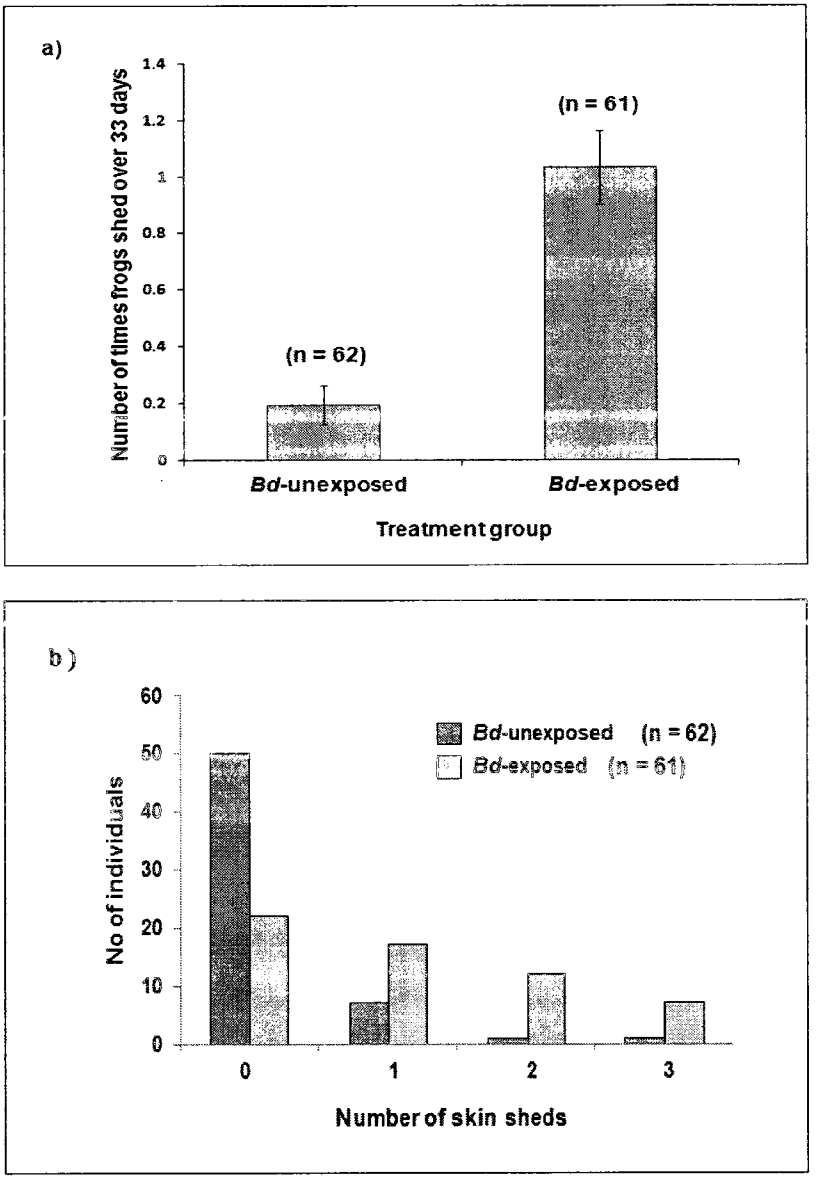

Figure 4. **(a)** Mean (± SE) number of times northern leopard frogs (*Lithobates pipiens*) pre-exposed to pesticides (atrazine, glyphosate, control) shed their skin during the time (33 days) they were subsequently exposed to *Batrachochytrium dendrobatidis* (*Bd*) ($p <$ 0.0001). **(b)** Number of northern leopard frogs that shed their skin 0, 1, 2 or 3 times during exposure to *Bd*. Frogs shown in (a) and (b) are pooled across the pesticide treatments.

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Appendix 1. List of abbreviations.

ae	Acid equivalent (of glyphosate)
AMP	Antimicrobial skin peptides
AMPA	Aminomethylphosphonic acid
ATV	*Ambystoma tigrinum* virus
Bd	*Batrachochytrium dendrobatidis*
CCAC	Canadian Council on Animal Care
CCME	Canadian Council of Ministers of the Environment
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
CTRV	Chronic toxicity reference value
HPLC	High performance liquid chromatography
HSI	Hepatosomatic Index
LGA	Granuloma in the liver
LMA	Melanomacrophage aggregate in the liver
MA	Melanomacrophage aggregate
MS-222	Tricaine-methanylsulfonate
N/L	Neutrophil to lymphocyte ratio
NOEC	No-observed-effects concentration
OIE	World Organization for Animal Health
OSI	Organosomatic Index
PCR	Polymerase chain reaction
POEA	Polyethoxylated tallow amine
RBC	Red blood cell
SMA	Melanomacrophage aggregate in the spleen
SSI	Splenosomatic Index
SVL	Snout-vent length
TFA	Tri-fluoroacetic acid
WBC	White blood cell

Appendix 2. Product information

1. 1.84L plastic containers: Small Pal Pens; Rolf C. Hagen Inc., Montreal, QC.
2. Crickets: Mirdo Importations Canada Inc., Montreal, QC.
3. P-free $CaCO_3$ powder: Repti-Cal supplement for reptiles and amphibians; Mirdo Importations Canada Inc., Montreal, QC.
4. Aatrex® Liquid 480, Syngenta Crop Protection Canada Inc., Guelph, ON.
5. Roundup® Original, Monsanto Canada Inc., Winnipeg, MA.
6. Mason jars: 1L canning jars, square-bodied, regular mouth, 12 per case; Canadian Tire, Toronto, ON.
7. Fiberglass screening: Saint-Gobain fiberglass screening, black; Canadian Tire, Toronto, ON.
8. MS-222: Tricaine-methylsulfonate, Syndel Laboratories Ltd, Vancouver, BC.
9. Tryptone powder: product number T7293, Sigma-Aldrich Canada Ltd., Oakville, ON.
10. 1% tryptone agar: 10 g tryptone powder + 10 g agar / 1L distilled water. (Agar: product number A1296, Sigma-Aldrich Canada Ltd., Oakville, ON).
11. Penicillin-streptomycin: product number P4333, Sigma-Aldrich Canada Ltd., Oakville, ON.
12. Blood staining kit: Protocol Hema-3® manual staining kit, Fisher Scientific, Ottawa, ON.