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Long term effects of carbaryl exposure on antiviral immune responses in *Xenopus laevis*

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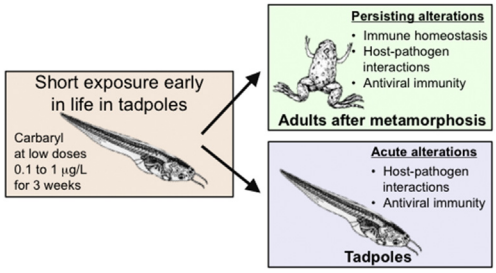
HIGHLIGHTS

- We examined the effects of low doses of carbaryl on *Xenopus* antiviral immunity.
- At higher doses carbaryl is toxic and accelerates tadpole development.
- Carbaryl impairs the tadpole innate immune response to FV3 infections.
- Carbaryl exposure of adults alters the FV3-induced IFN-I response.
- Developmental exposure to carbaryl induces lasting changes in adult antiviral immunity alterations.

GRAPHICAL ABSTRACT



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ABSTRACT

Water pollutants associated with agriculture may contribute to the increased prevalence of infectious diseases caused by ranaviruses. We have established the amphibian *Xenopus laevis* and the ranavirus Frog Virus 3 (FV3) as a reliable experimental platform for evaluating the effects of common waterborne pollutants, such as the insecticide carbaryl. Following 3 weeks of exposure to 10 ppb carbaryl, *X. laevis* tadpoles exhibited a marked increase in mortality and accelerated development. Exposure at lower concentrations (0.1 and 1.0 ppb) was not toxic, but it impaired tadpole innate antiviral immune responses, as evidenced by significantly decreased TNF-α, IL-1β, IFN-I, and IFN-III gene expression. The defect in IFN-I and IL-1β gene expression levels persisted after metamorphosis in froglets, whereas only IFN-I gene expression in response to FV3 was attenuated when carbaryl exposure was performed at the adult stage. These findings suggest that the agriculture-associated carbaryl exposure at low but ecologically–relevant concentrations has the potential to induce long term alterations in host-pathogen interactions and antiviral immunity.

1. Introduction

Aquatic vertebrates are increasingly exposed to a large number of water pollutants associated with agriculture (Gibbs et al., 2009; Serpa et al., 2014; Smalling et al., 2015). These pollutants are detectable in most aquatic habitats, but their long-term effects at environmentally relevant levels on immune function remains unclear. Aquatic vertebrates are also exposed to circulating viruses and other pathogens, and there is some evidence suggesting associations between exposures to pollutants and greater susceptibility to circulating pathogens (Brown et al., 2013; Christin et al., 2003; Kerby and Storfer, 2009). We have established the amphibian *Xenopus*

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laevis and the ranavirus Frog Virus 3 (FV3) as a reliable experimental platform for evaluating the effects of common waterborne pollutants on antiviral immune defenses across the lifespan.

Ranaviruses are contributing to the worldwide amphibian decline. Notably, ranavirus infections have not only become more prevalent over the past decade, but have also markedly increased in host range in amphibians and other ectothermic vertebrates. Indeed, besides more than 100 species of amphibians, there are a growing number of reptile and fish species reported to be infected by ranavirus pathogens (Chinchar et al., 2009; see eBook (Gray and Chinchar, 2015)). While emerging infectious diseases caused by ranavirus pathogens are concerning for biodiversity and aquaculture, they also raise questions about efficacies of antiviral immunity in amphibians in particular and aquatic vertebrates in general. Given the deleterious effects of environmental pollutants on animal development and physiology, it is important to evaluate more thoroughly their possible impact on immune function, and thus susceptibility to viral pathogens.

Pesticides have been proposed to represent a major cause of amphibian declines (Davidson et al., 2001; Davidson, 2004; Fellers and Drost, 1993; Houlahan et al., 2000). However, to date experimental evidence of the role played by environmental pollutants remains scant. In part this is due to the complexity of interactions between contaminants, pathogens and hosts. Some field studies have established correlations between the amounts of pesticides and the decline of frog species (Carey and Bryant, 1995). Other studies have reported cumulative and interactive toxic effects of pesticides at low concentrations on amphibian development and survival (Boone and Bridges-Britton, 2006; Hayes et al., 2006; Relyea, 2009). Concerning the immune function, we have previously shown that water containing the herbicide atrazine at subtoxic level induces long lasting antiviral immune deficits in *X. laevis* (Sifkarovski et al., 2014). The interpretation was that exposure even at low levels during early life can result in persistent effects that negatively impact immune defenses to pathogens such as FV3. To further address this possibility, we have examined the effects of another prominent water contaminant, the insecticide carbaryl.

Carbaryl (1-Naphthyl-*N*-metylcarbamate) is a pesticide used worldwide, and it has a tendency to reach water sources via agricultural runoffs. The U.S. Environmental Protection Agency (EPA) considers a maximum contaminant level for carbaryl at 40 parts-per-billion (ppb, 40 μg/L). Notably, the yearly average of carbaryl levels found in the Rochester NY area, is 0.3 ppb (http://www.ewg.org/tap-water/whatsinyourwater/2021/NY/NewYork/Carbaryl/) but can be as high as 1 ppb, and in regions with intensive pesticide drifts (e.g., Colorado), levels up to 16.5 ppb have been reported (cdpr.ca.gov/docs/emon/pubs/fatememo/carbaryl.pdf). Exposure to carbaryl has been linked to mutagenesis, disruption of hormone function (endocrine disruptor), and alteration in the immune system of humans (npic.orst.edu/factsheets/carbgen.pdf). Carbaryl can also be toxic to a variety of non-targeted species (birds, fish and amphibians). In anuran amphibians, the minimum lethal concentration is estimated to be 4.8 mg/L (4.8 ppm) (Davidson et al., 2007). However, aquatic animals such as amphibians are continuously exposed to water pollutants and therefore, may endure adverse health effects of carbaryl even at lower doses, which may become more critical during infectious diseases. Indeed, carbaryl appears to be more toxic for fish (rainbow trout) with an LC_{50} of 1.4 mg/L (1.4 ppm) for a 96 h exposure time. Exposure to a sublethal dose of carbaryl has been shown to reduce host skin peptide defenses of several anurans species, which may increase susceptibility to infection by skin pathogens such as the chytrid fungus (Batrachochytrium dendrobatidis) (Davidson et al., 2007; Schadich et al., 2009; Buck et al., 2015). To examine in more detail the potential immunomodulatory effects of carbaryl, we took advantage of the *X. laevis*/FV3 model system (Chen and Robert, 2011; De Jesus Andino et al., 2012; Morales et al., 2010). Specifically, we tested the hypothesis that developmental exposure at the tadpole stage to levels of carbaryl in the water that are at or below current measured levels in the environment results in alterations of antiviral immunity later in life, thus increasing susceptibility to pathogens such as FV3.

2. Materials and methods

2.1. Animals

All outbred *Xenopus laevis* tadpoles and adult frogs were acquired from the *X. laevis* research resource for immunology at the University of Rochester (http://www.urmc.rochester.edu/mbi/resources/Xenopus/). For tadpole survival and gene expression experiments, stage 50 and 56 tadpoles were used, respectively (Nieuwkoop and Faber, 1967). One-year-old frogs were used for all adult experiments. All animals were handled in accordance with stringent laboratory and University Committee on Animal Research regulations (Approval number 100577/2003-151).

2.2. Carbaryl exposure

Highly purified carbaryl (Chem Service. West Chester, PA) was dissolved in DMSO as an initial stock solution from which subsequent working solutions were prepared. Two-week old (developmental stage 50; (Nieuwkoop and Faber, 1994)) tadpoles (10–25 individuals per group depending of the experiment) were exposed for 3 weeks to 0.1, 1.0 or 10.0 ppb carbaryl in 4 L containers (Fig. 1A). A concentration of 0.5% DMSO equivalent to the highest dose of carbaryl was used as a control in all experiments. The water and fresh carbaryl (or DMSO for control) were changed every week for each treatment group. All tadpoles were then transferred into 4 L containers of clean water for recovery during one week to minimize possible stress due to the treatment. Survival following exposure to carbaryl was determined on groups of 25 individuals, whereas effects on developmental stages were monitored every day until metamorphosis completion for groups of 10 individuals. For adult treatment, one-year old outbred adults were exposed to 0.1, 1.0 or 10.0 ppb carbaryl in 2 L of water for 3 weeks.

2.3. Frog Virus 3 stocks and infection

Fathead minnow cells (FHM; American Type Culture Collection, ATCC No. CCL-42) and baby hamster kidney cells (BHK-21, ATCC No. CCL-10) were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), streptomycin (100 μg/mL), and penicillin (100 U/mL) with 5% CO2 at 30 °C and 37 °C, respectively. FV3 was grown using a single passage through FHM cells or BHK-21 cells and was subsequently purified by ultracentrifugation on a 30% sucrose cushion. Plaque assays on a FMH or BHK-21 cell monolayers were used to quantify FV3. Tadpoles were infected by intraperitoneal (i.p.) injection with 1×10^4 plaque forming units (PFU) of FV3 in 10 μL aliquots of amphibian phosphate buffered saline (APBS). Post-metamorphic one year-old frogs were infected by i.p. injection of 1×10^6 PFU in 100 μL. For all i.p. infections, uninfected control animals were mock-infected (i.p.) with an equivalent volume of APBS. One to 6 days post-infection (dpi), animals were euthanized using buffered MS-222 for kidney extraction (Fig. 1, bottom panel).

2.4. Quantitative gene expression analyses

RNA and DNA were extracted from frog kidneys using Trizol reagent, following the manufacturer's protocol (Invitrogen). Total