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Effects of imidacloprid on *Rana catesbeiana* immune and nervous system

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HIGHLIGHTS

- Imidacloprid did not cause neurological toxicity in tadpoles.
- Imidacloprid did not prevent tadpoles from producing an immune response.
- Injection with keyhole limpet hemocyanin (KLH) caused an isotype switch in tadpoles.
- Post-injection with KLH caused a sharp increase in tadpole mortality.

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ABSTRACT

Imidacloprid (IMD), a neonicotinoid, is generally considered to be of low toxicity in vertebrates. However, the inhibition of acetylcholine (ACh) receptors can have a profound effect on both the immune and nervous system due to the anti-inflammatory effects of ACh. Vertebrates, such as amphibians, might be affected by IMD because they breed in wetlands where the concentration of IMD is high. In our study, we experimentally exposed *Rana catesbeiana* tadpoles to environmentally relevant IMD and then quantified the ACh and antibody to non-replicating antigens. We hypothesized that IMD exposure would result in higher AChE and antibody levels. We completed a factorial experiment in which tadpoles were divided into four groups, two of which were exposed to 100 ng/L of IMD. After five weeks, two groups were injected with the novel antigen keyhole limpet hemocyanin (KLH) and two injected with a control. Three weeks later, tadpoles were euthanized and blood samples collected. At 100 ng/L, IMD exposure did not cause a significant difference in AChE levels or KLH-specific IgY antibodies. However, tadpoles injected with KLH had slightly higher levels of AChE. In addition, we saw a trend in total IgM with higher levels in tadpoles exposed to IMD. While we found no effect of IMD at 100 ng/L on antibody response to a novel, non-replicating antigen nor on ACh production, further research is needed to determine if higher concentrations of IMD or parasite infection can influence development of *R. catesbeiana*.

1. Introduction

In the past three decades a new group of insecticides, known as neonicotinoids, was developed and introduced to the agriculture industry. Neonicotinoids are used primarily as plant systemic insecticides. In, 1991, imidacloprid (IMD), a specific neonicotinoid, was introduced to the United States. IMD (IUPAC name 1-[(6-chloropyridin-3-yl) methyl]-N-nitro-4, 5-dihydroimidazol-2-amine (Suchail et al., 2000) became a widely used, highly effective insecticide. IMD causes a nearly irreversible blockage of post-synaptic nicotinic acetylcholine (ACh) receptors in the central nervous system (CNS) of insects after ingestion or direct contact (Mason et al., 2013). IMD binds to ACh receptors with a greater affinity and act as 'false neurotransmitters' (agonists). This blockage causes ACh to accumulate in the synaptic cleft consequently causing neurological toxicity (Gibbons et al., 2015). In order to reverse the blockage more enzymes are synthesized (Fulton and Key, 2001). IMD is a colorless, odorless crystal, and a General Use Pesticide, classified by the EPA as a class II/III agent (Starner and Goh, 2012). IMD is the largest selling insecticide in the world due to its most common uses as either a seed or soil treatment on a number of agricultural products including rice, cereal, maize, potatoes, vegetables, sugar beets, fruits, cotton, hops, and turf (Badgujar et al., 2013). When used as a seed dressing, IMD migrates

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rapidly from the stem to the leaf, and then into the flower and pollen of the plant (Mason et al., 2013). IMD is regularly used for controlling sucking insects, soil insects, termites, some chewing insects, and as a topical flea control treatment on domestic pets, but has affected a number of non-target arthropods including honeybees and parasitic wasps (Suchail et al., 2000), which raises the possibility for non-target vertebrates to also be influenced by IMD.

IMD is relatively low in toxicity in vertebrates, likely due to structural differences in nicotinic receptors between the targeted insects and vertebrates (Badgujar et al., 2013). However, studies suggest that both chronic and acute exposure to IMD can increase the likelihood of respiratory diseases, neurological dysfunctions, cancers, and reproductive disorders in humans (Duzguner and Erdogan, 2010). Yeh and colleagues examined a case of IMD-poisoning after alcohol consumption in which the patient had acute multiple organ failure including kidney and lung, and hypotension and metabolic acidosis (Yeh et al., 2010). Furthermore, a study in Taiwan revealed an increased number of neonicotinoid poisoning cases in humans, including an attempted suicide via an ingestion of 40 mL in 20 min of a Taiwan insecticide with 9.6% IMD as the active ingredient (Lin et al., 2012). With the recent spike in neonicotinoid-poisoned patients, investigations have found that in humans, IMD stimulates acetylcholine receptors and interferes with the transmission of neuronal impulses through fatigue (Lin et al., 2012). This effect of IMD on the CNS results in dizziness, drowsiness, disorientation, and coma. At high enough concentrations or prolonged exposure, the effects on the autonomic nervous system include diaphoresis, mydriasis, tachycardia, and hypertension which can lead to coronary spasm and cardiac ischemia, followed by nervous system paralysis. Furthermore, cardiac depression, neurological depression, and corrosive injury from solvents within the insecticide causes edema of the airway and the resultant inflammation can cause respiratory failure (Lin et al., 2012).

In addition to nervous system effects, the inhibition of ACh receptors can have a profound effect on an organism's immune response. Macrophages contain nicotinic receptors that bind ACh to terminate production of pro-inflammatory cytokines (Duzguner and Erdogan, 2010). As ACh receptors are blocked by IMD, so is the anti-inflammatory effects of acetylcholine on macrophages. Furthermore, the residual components of IMD, imidacloprid urea, 6-chloronicotinic aldehyde, 6-chloro-N-methylnicotinacidamide, and 6-chloro-3-pyridyl-methylethylenediamine (Moza et al., 1998), can lead to immunosuppression in animals either directly or through participation of stress mechanisms and the neuroendocrine system (Gawade et al., 2013). Immunosuppression can alter life-span, increase susceptibility to infectious diseases, and decrease immune response to foreign antigens (Gawade et al., 2013). Mohany et al. examined the immunotoxic effects of IMD on male albino rats and found a significant increase in total leukocyte count of rats after oral administration of IMD. Furthermore, the same study investigated changes in serum immunoglobulins (Igs) and found IMD induced a significant increase in IgG, but not IgM (Mohany et al., 2011). After four weeks of IMD oral exposure the histological structure of the spleen, thymus, and liver show significant differences to that of non-IMD exposed rats (Mohany et al., 2011).

The tremendous use of IMD in recent years may have added to its soil persistence and storage as well as water contamination. Water contamination can occur during, and following, many of the agricultural application methods of IMD. Dust can settle into water, spray droplets can drift into nearby water, runoff from treated fields can be contaminated, coated seeds can leach into the water, and lastly, decomposition of treated plants can reintegrate IMD into the soil and soil water (Kreutzweiser et al., 2007). Further, the USA EPA has classified IMD as highly toxic to aquatic invertebrates (Starner and Goh, 2012). Thus, amphibians may be affected by IMD throughout their life, each in their fully aquatic larval stages, or as terrestrial adults. Tadpoles are particularly susceptible to the effects of these pollutants in their water due to their thin, porous skin (Mason et al., 2013). Amphibians, especially tadpoles, are thought to be indicator species of overall environmental health due to their unique niche at the edge of the aquatic-terrestrial ecosystems (Mason et al., 2013). Sequentially, in 1999, eight years after IMD was introduced to the United States, two novel pathogens, the chytrid fungus (*Batratachochytrium dendrobatidis*) and the *ranavirus*, devastated the world's amphibian population and continue to now. Consequently, this raised the question of whether pesticide exposure may be weakening amphibian immune defenses, and thus increasing their susceptibility to pathogens.

As a result, this study is aimed at observing the effects of IMD on the nervous system in the American Bullfrog, *Rana catesbeiana*, by measuring acetylcholinesterase (AChE) levels after exposure to IMD at an environmental relevant concentration (100 ng/L) for five weeks and examining *R. catesbeiana* immune response after being exposed to a novel antigen, keyhole limpet hemocyanin (KLH), by measuring antibody response. By using a non-replicating antigen, we can isolate the effects of IMD on the immune response from the potential immunomodulating effects of a replicating parasite. In toxicology, AChE can be used as a significant indicator of neurological toxicity in both vertebrates and invertebrates (Yadav et al., 2009). AChE catalyzes the hydrolysis of acetylcholine into choline and acetic acid at the synaptic junctions. Thus, AChE helps facilitate nerve impulse transmission between cholinergic neurons by controlling the flow of ionic currents at the synapse and neuromuscular junction. AChE seems to be effective in identifying the biological effects of several neurotoxic contaminants in aquatic habitats (Yadav et al., 2009). In previous studies, the inhibition of AChE by a xenobiotic compound has been used to diagnose the toxicity of two pesticides: organophosphates and carbamates. Therefore, AChE can also be used to indicate toxicity of neonicotinoid poisoning despite the relative small amount of previous reports. Immunization with a novel agent ensures our results are not confounded by variable degrees of prior immunity to the immunogen (Smith et al., 2004). KLH is a protein found in the marine mollusc (*Megathura crenulata*), and thus, the tadpoles are unlikely to be exposed to it prior to our immunization. It is simple and safe, and ensures activation of the humoral immune response without other confounding factors (Smith et al., 2004). Based on IMD's nervous system response in vertebrates and insects, we hypothesized that higher levels of AChE and a lower antibody production will be found in tadpoles exposed to IMD than in tadpoles never exposed to IMD.

2. Methods

Rana catesbeiana tadpoles were purchased from a licensed collector in West Virginia between the developmental stages 20–25 (Gosner, 1960), and shipped to Millikin University in Decatur, IL. They were kept at 22 °C with natural lighting in an open lab at Millikin University. For this factorial experiment, tadpoles were randomly divided into four separate groups (IMD-/KLH-; IMD+/KLH-; IMD-/KLH+; IMD+/KLH+). Each group consisted of ten 0.5 L plastic containers with four tadpoles per container (40 tadpoles per group). Half of the water was changed every two to three days during the first two weeks under controlled conditions prior to starting the experiment to allow for the tadpoles to acclimate to the conditions. The tadpoles were fed ¼ of a Spirulina algae based bottom feeder tablet at every water change. The experimental treatments were initiated after the two-week acclimation period, and lasted for eight weeks. During the experimental phase, their