THE EFFECTS OF NEONICOTINOID EXPOSURE ON EMBRYONIC DEVELOPMENT AND ORGAN MASS IN NORTHERN BOBWHITE QUAIL

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Since their emergence in the early 1990s, neonicotinoid use has increased exponentially to make them the world's most prevalent insecticides. Although there is considerable research concerning the lethality of neonicotinoids, their sub-lethal and developmental effects are still being explored, especially with regards to non-mammalian species. The goal of this research was to investigate the effects of the neonicotinoid imidacloprid on the morphological and physiological development of northern bobwhite quail (*Colinus virginianus*). Bobwhite eggs (n = 650) were injected with imidacloprid concentrations of 0 (sham), 10, 50, 100, and 150 grams per kilogram of egg mass, which was administered at day 0 (pre-incubation), 3, 6, 9, or 12 of growth. Embryos were dissected on day 19 when they were weighed, staged, and examined for any overt structural deformities. Embryonic heart, liver, lungs and kidneys were also weighed and preserved for future use. Treated embryos exhibited increased frequency of severely deformed beaks and legs, as well as larger hearts and smaller lungs at the higher dosing concentrations. Some impacts are more pronounced in specific dosing periods, implying that there may be critical windows of development when embryos are highly susceptible to neonicotinoid exposure. This investigation suggests that imidacloprid could play a significant role in chick survival and declining quail populations in treated regions of the country.

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Introduction

The primary goal of this research was to examine how embryonic exposure to neonicotinoid insecticides impacts the physiology and development of northern bobwhite quail (*Colinus virginianus*). Neonicotinoids are a class of pesticides introduced in the early 1990s which have since become the most widely used insecticides in the world, and which are applied to a variety of crop types (Goulson, 2013; Millar and Denholm, 2007). Although there has recently been intense interest in the effects of neonicotinoids on non-target organisms such as pollinating insects (Blacquière et al., 2012; Moise et al., 2003) and mammals (Gawade et al., 2013), there is still relatively little information regarding the impact on birds. Bobwhite quail are a species of particular concern in this case, since they are known to be found in areas where the insecticides are routinely applied (Dimmick, 1992) and have suffered a severe population decline in recent decades (*Upland Urgency: The Fight against Bobwhite Quail Decline*, 2011). This work has helped to fill knowledge gaps in this area by testing the effects of the neonicotinoid imidacloprid on quail in ovo, during the especially sensitive life history period of embryonic development.

Furthermore, this study has approached the problem in a unique way by focusing primarily on the sub-lethal effects of insecticide exposure rather than exclusively on mortality. As emphasized in the subsequent literature review, sub-lethal and developmental impacts of pesticide exposure have been largely neglected in previous

work, despite evidence suggesting that these influences can have significant impacts on long-term survival (Bennett and Etterson, 2006; Mineau, 2002). Sub-lethal effects have been notoriously difficult to measure in the field due to unpredictable environmental variables and high rates of scavenging and predation (Vyas, 1999), but this investigation has sought to reproduce them in a controlled laboratory environment. To this end, embryos were exposed to a range of neonicotinoid concentrations during several stages of incubation and were evaluated in terms of embryonic and organ masses, developmental progress, and the presence or absence of overt anatomical deformities. The results indicate that exposure to neonicotinoids affects quail development in ways that threaten their survival both pre and post hatch.

An understanding of how neonicotinoid use affects quail is essential in determining their potential contribution to declining northern bobwhite quail populations. This research has contributed to that understanding by evaluating the pesticide's sub-lethal impacts on eggs and embryos, which might be exposed to pesticides directly from the environment or by transfer from the laying hen. Previous work has indicated that embryos exposed to toxicants may be subject to altered organogenesis (Antkiewicz et al., 2005; Bruggeman et al., 2003; Kabuto et al., 2004) and anatomical malformations (Gawade et al., 2013; Seifert and Stollberg, 2005), and that specific structures are more or less susceptible to exposure during different developmental periods (Bunn et al., 2001; Burggren and Reyna, 2011). Here, we have tested the hypotheses that the hearts, livers, lungs and kidneys of exposed embryos will exhibit significant mass variation; that mortality and prevalence of deformity will be higher among exposed embryos; and that there will be critical windows of development for each organ when mass variation is more likely to occur. A review of

relevant literature will provide background on the history of neonicotinoids, their mode of action, and their documented effects on birds and other non-target organisms to give context to the results of these experiments.

Literature Review

History of Neonicotinoids

Neonicotinoid insecticides first became commercially available in the early 1990s and are now the most frequently used pesticide in the world (Gibbons et al., 2014; Goulson, 2013). Their success is attributable to several factors (Simon-Delso et al., 2014). Flexibility is one reason for their surge in popularity: neonicotinoids can be used on a variety of crop types including vegetables, fruits and cereals, and they may be applied as either a seed coating or a foliar spray. Neonicotinoid use is not restricted to crop production, having found additional markets in veterinary medicine as flea and tick protectants and in fish farming as invertebrate pest control (Simon-Delso et al., 2014). A lack of neonicotinoid resistance among pest species also contributed to their initial adoption, but fervent use over the past 20 years has made the chemical less effective on its target pest populations (Bass et al., 2015; Elbert et al., 2008). Probably the most crucial contributing factor to neonicotinoids' success is their perceived safety to consumers. The insecticide is designed to be lethal to insects while posing little to no threat to mammals (Jeschke et al., 2013); this fact, coupled with neonicotinoids' ease of use and diversity in application options, makes them a more appealing pest control option than previously

utilized chemicals, such as organophosphates, carbamates, and pyrethroids, which had proved hazardous to both the environment and human health.

The most common varieties of neonicotinoids currently used in the United States and United Kingdom are imidacloprid and clothianidin, respectively. Other popular forms include acetamiprid, thiacloprid, dinotefuran, and nitenpyram (Casida, 2011). Neonicotinoids appear in the consumer market under various brand names, such as Admire ®, Merit ®, Gaucho ®, and Advantage ®, which are manufactured by Bayer Crop Science (Blacquière et al., 2012; Goulson, 2014). Although they have proven effective on a variety of insect types, they are primarily used to guard against Hemipterans (Jeschke et al., 2013). In the U.S. and in Texas, specifically, imidacloprid-based seed coatings and sprays are frequently used to treat corn, potatoes, and cotton. In the year 2010, more than 49,000 pounds of imidacloprid were applied to cotton crops in the United States alone (United States Department of Agriculture, 2011).

The public's outlook on neonicotinoid insecticides has changed in recent years, and the chemical's reputed low environmental impact is being re-evaluated. European nations have taken measures to restrict neonicotinoid use in an attempt to protect declining honeybee populations that may be threatened by the insecticide. In 2013, the European Food Safety Authority established a two year moratorium on neonicotinoid use in flowering crops (Barroso, 2013). However, neonicotinoids are still permitted on non-flowering crop types in Europe, and their use remains completely unrestricted in other parts of the world (Goulson, 2014). Concerns have also prompted worldwide investigations into the effects of neonicotinoids and other systemic pesticides on all non-target species (Gross, 2014; Mineau and Palmer, 2013; Moser and Obrycki, 2009;

Whitehorn et al., 2012). Reports also indicate that the loss of key ecosystem services, such as pollination by honeybees, may make neonicotinoids a net loss economically as well as environmentally (Blacquière et al., 2012; Gross, 2014).

Mode of Action and Metabolic Pathway

There are two primary options for the application of neonicotinoids: foliar spray and seed coatings (Goulson, 2013). Foliar spray is an insecticide solution applied directly to the surface of the crop, while seed coatings are a solid form of the pesticide that is applied prior to sowing. For both application methods, the pesticide dissolves in water and is readily absorbed into plant tissues, where it effectively guards against feeding insects even at concentrations as low as 5–10 ppb (Goulson, 2013; Simon-Delso et al., 2014). The systemic nature of seed coatings ensures that all parts of the plant are protected starting from germination, and this fact, coupled with their ease of use, makes coated seeds the most frequently used method of application (Gross, 2014). Coated seeds are so thoroughly integrated into modern agriculture that finding seeds without neonicotinoid treatment can be difficult. In North America for example, almost all of the seeds planted (except in organic farming) have insecticidal coatings, and in many cases there are actually no untreated seeds available for purchase (Simon-Delso et al., 2014).

Despite their propensity to translocate into plant tissues, many of the neonicotinoids that are applied never make it into the target crop and instead contaminate the surrounding environment (Goulson, 2014). Only about 5% of the chemical in a coated seed is actually taken up by the plant, 1% blows away during sowing, and the remaining 94% seeps into the surrounding soil and water. Although neonicotinoids are relatively

short-lived in direct sunlight, in soil and water mediums their half-lives can easily exceed 1,000 days. This highly persistent nature coupled with repeated applications over multiple growing seasons ensures that neonicotinoid concentrations tend to increase in the environment over time (Goulson, 2014; Gross, 2014). Research has supported the accumulation hypothesis with water and soil samples containing neonicotinoid concentrations that far exceed the amounts necessary to control pests (Gibbons et al., 2014; Goulson, 2013; Hladik et al., 2014).

The selectivity of neonicotinoids to invertebrate pests is due to their unique chemical structure and differences in their affinity for vertebrate and invertebrate acetylcholine receptors. Neonicotinoids are agonists (chemicals that bind to and activate receptors) of insect nicotinic acetylcholine receptors (nAChRs) which are designed to mimic the effects of nicotine (Gotti and Clementi, 2004; Seifert and Stollberg, 2005). In vertebrates, nAChRs play important roles in synaptic transmission in both neuromuscular junctions and within the peripheral and central nervous system. Insects rely on ACh receptors far more heavily than mammals do (the insect nervous system has some of the densest concentrations of neuronal nAChRs), making them prime targets for insecticides (Millar and Denholm, 2007). Nicotine is highly poisonous to both vertebrates and invertebrates, while neonicotinoids are engineered to be selectively targeted to insects (Jeschke et al., 2013; Tomizawa and Casida, 2001; Yamamoto, 1999). This is due to differences in how insect and vertebrate nAChRs are structured: insect receptors have a cationic subunit which strongly attracts the negative nitro and cyano groups on neonicotinoid compounds (Millar and Denholm, 2007).

Although neonicotinoids are designed to be selectively toxic to invertebrates, their effects on mammals, birds and other organisms ultimately depend upon a number of different factors, the first being that the toxicity of a poison depends on three variables: the distribution and metabolization of the chemical in the body, the concentration of the chemical in relation to how many receptors are available for its binding, and the physiological effects resulting from the binding of those receptors (Henk and Sanchez-Bayo, 2011; Lu et al., 2003). The propensity of neonicotinoids to permanently bind insect nAChRs ensures that exposure time and sub-lethal effects are important considerations in determining their toxicity, especially with regard to aquatic invertebrates. Imidacloprid concentrations up to 75,000 times lower than acute LC50 values for cladocerans and ostracods have eliminated those species from aquatic mesocosms when applied over a two month period, and both *Baetis* and *Epeorus* mayflies have experienced reduced reproductive success at imidacloprid exposures as low as 100 ng/L. One stonefly species was similarly affected at a concentration 70 times below its lowest documented LC50 (Henk and Sanchez-Bayo, 2011). The effect of neonicotinoids affect bobwhite quail will also depend on how it is metabolized in the avian body, how the insecticide and its metabolites interact with quail physiology, and how duration of exposure alters the toxicological response.

The general metabolic pathway of neonicotinoids has been identified in previous research and is surprisingly consistent across vertebrate organisms, with species-specific differences occurring primarily in aldehyde oxidase (AOX) activity levels within the liver (Casida, 2011). Though there are several neonicotinoid varieties, all of them share the same basic molecular structure which, in the case of imidacloprid, can be subdivided into

the following 3 components: a chlorine-benzene complex, an imidazolidine moiety with 2 methylene substituents, and the electronegative N-nitro tip that is crucial for receptor selectivity (see Appendix for a structural diagram). Variations in substituents between the types of neonicotinoids imbue each chemical with its unique properties and products (Casida, 2011). When imidacloprid is metabolized within the body, enzymes act upon each of the three groups in two phases. During phase 1 metabolism, a suite of enzymes collectively referred to as CYP450 work to introduce or unmask a polar group on the molecule in order to increase its solubility and therefore facilitate its excretion from the body (Klaassen and III, 2010). Phase 1 for imidacloprid involves nitro reduction by CYP2D6 and olefin formation by CYP3A4. The second phase of toxin metabolism prepares the material for excretion by covalent attachment of a functional group to increase hydrophilicity (Klaassen and III, 2010). In imidacloprid, this process may occur via glucuronidation, methylation, acetylation, or sulfonation. Although metabolization is generally thought to decrease chemical toxicity, in some cases metabolites have equal or even greater toxicity than the parent compound (Fossen, 2006; Simon-Delso et al., 2014).

In the case of neonicotinoids, there are two types of metabolites that are of primary concern: substances that, like the parent compound, act as nACh receptor agonists, and those that have secondary toxic effects. Desnitro and descyano products from the metabolization of imidacloprid continue to stimulate acetylcholine receptors, but do so with increased affinity for mammalian receptors and with a potency comparable to that of nicotine (Casida, 2011). Secondary effects of neonicotinoid metabolites are not as thoroughly studied but have been known to include hepatotoxicity, cancer, and antinociception in mice (Casida, 2011; Tomizawa et al., 2001). Although neonicotinoids

themselves are alleged to be relatively non-toxic to vertebrates, evidence suggests that vertebrate metabolic processing may produce substances that are both more effective as receptor agonists and induce chemical injury in the liver.

Implication in the Bee Decline

Neonicotinoids have been the subject of much recent debate due to their alleged role in the decline of honeybee populations (Goulson, 2013; Reynard, 2012). There is an inherent difficulty in designing a chemical that will kill pest insects while leaving beneficial insects like honeybees alone. Neonicotinoids are especially problematic in this regard because their oral toxicity is 3-5 times greater than their contact toxicity, meaning that even trace amounts in pollen can endanger bee colonies that use it for food (Fossen, 2006). Imidacloprid has been detected in pollen at concentrations between 0.9 and 3.1 µg/kg and in plant secretions that bees ingest for food (Blacquière et al., 2012), with oral exposure being more toxic than direct contact. Honey and beeswax has also been found to contain imidacloprid at lower concentrations. Lab studies indicate that chronic exposure results in worker deaths and that the aforementioned neonicotinoid metabolites can also be highly toxic to bees (Blacquière et al., 2012).

Research suggests that neonicotinoids' sub-lethal effects on immunocompetence and behavior may be a greater contributor to the collapse of bee colonies than direct mortality (Blacquière et al., 2012; Di Prisco et al., 2013; Reynard, 2012). Recent work has demonstrated that clothianidin interferes with NF-κB signaling in honeybees and increases their susceptibility to viral infections (Di Prisco et al., 2013). The insecticide alters gene transcription to upregulate Amel\LRR, which impedes the NF-κB pathway and

weakens the immune response. Behavioral symptoms of exposure include incoordination, trembling and seizures as well as difficulty learning, memorizing and relaying patterns—all of which are necessary for bees to communicate with hive mates (Blacquière et al., 2012; Reynard, 2012). Reproduction is also impaired through delayed hatching and maturation times and decreased larvae production (Blacquière et al., 2012).

Bees are not the only non-target invertebrate to feel the effects of neonicotinoids, and evidence indicates that biomagnification is a concern as well. Neurological symptoms have been observed in coccinellid larvae (*Harmonia axyridis*) after feeding on seedlings grown from treated seeds (Moser and Obrycki, 2009). In rice fields treated with imidacloprid, there were smaller populations of high-trophic level insects versus control fields, and the treated paddies suffered from algal blooms due to the extirpation of benthic organisms that normally feed on phytoplankton (Sánchez-Bayo and Goka, 2006).

Northern Bobwhite Quail as a Species of Interest

The northern bobwhite quail, *Colinus virginianus*, is an important species both ecologically and economically. Bobwhites require a variety of cover types for feeding, nesting, and brood rearing, and so habitat that suits them typically supports a high diversity of other grassland species (Brennan et al., 2005; Dimmick, 1992). Habitat management aimed at boosting bobwhite populations can also benefit a number of prairie songbird species (Northern Bobwhite (*Colinus virginianus*), 1999). Northern bobwhites are also an important food source for many terrestrial and aerial predators (Guthery, 2006); this fact, coupled with their high sensitivity to environmental variables, makes them an invaluable indicator species for grassland ecosystems. Bobwhites have economic

significance as well, since quail hunting is a considerable source of state revenue, sometimes comparable to grazing leases. Among quail enthusiasts, the average amount spent on quail hunting in 1999 exceeded $10,000 per individual, and sales of non-resident hunting licenses have increased more than 260% since 1987 (Brennan et al., 2005; Johnson et al., 2012).

Northern bobwhite population sizes have decreased by more than 80% since the 1960s (Sauer et al., 2013; Texas Parks and Wildlife Department, 2011), with several factors implicated as potential causes. Habitat loss due to urbanization and agricultural expansion is frequently cited for causing population declines in prairie bird species, including bobwhite quail (Brennan et al., 2005; Mineau and Whiteside, 2013). Much of the remaining habitat deemed suitable for quail has been severely fragmented, resulting in smaller populations that are more susceptible to disease and sudden environmental changes (Brennan, 2007; Guthery, 2006). Other research suggests that bobwhite reproduction is significantly depressed during drought conditions that induce heat stress (Guthery et al., 2002; Reyna, 2010). Populations are further stressed by high rates of predation from bobcats, coyotes, birds of prey, insects (which may feed on eggs or newly hatched chicks) and human hunters (Guthery, 2006). With so many factors exerting pressure on bobwhite populations that are already in decline, it is crucial to determine how neonicotinoids might be influencing quail survival.

Bobwhites are heavily reliant on high rates of reproduction for maintaining healthy populations, as evidenced by their r-selective life history. The average lifespan of adult birds is notably short at an estimated 6 months with an exceptionally high turnover rate of 80% annual adult mortality (Brennan, 1999; Guthery, 2006). Although adults rarely live

more than a year, they compensate with their impressive reproductive potential. Hens lay 12–14 eggs per clutch and can make multiple nesting attempts during a breeding season (Brennan, 1999), with nesting success—as defined by the fraction of nests which successfully hatch at least one egg—typically between 30 and 40% (Klimstra and Roseberry, 1975; Potter et al., 2011). Low survival in adults means that poor performance during a single breeding season can have a noticeable impact on bobwhite population abundance. Therefore, any factor that influences reproductive potential, whether it impacts clutch size, egg viability, embryonic development or mating behavior, may further jeopardize the already diminished quail populations.

Potential Exposure to Neonicotinoids

There are several ways that birds might be exposed to neonicotinoid insecticides, and the path of exposure can be critically important in determining toxicity. Driver et al. (1991) examined bobwhite susceptibility to an organophosphate pesticide through several pathways, including feeding, dermal absorption, preening, and respiration. The impact of each pathway was evaluated through analysis of brain cholinesterase activity in affected individuals. It was found that inhalation was the primary exposure route 1 hour after a foliar application, but preening accounted for most pesticide uptake 4 hours later. Ingestion of contaminated food accounted for 10–20% of the impact but was secondary to dermal exposure 8 hours after application (Driver et al., 1991). All of these are ways that bobwhites might come into contact with neonicotinoids in their environment, but the field relevance of each pathway will depend on whether the insecticide is applied as a spray or a seed coating in a given area.

The inhalation and dermal contact pathways are especially relevant in areas that are treated with neonicotinoid foliar sprays; it is therefore important to determine if bobwhites use this habitat and how it might put them at risk. Puckett et al. (1995) examined this particular scenario revealing how bobwhites utilize agricultural fields for nesting and brood rearing. They found that habitat use and nesting success was low in soybean fields early on in the growing season, but as the plants matured, they provided a 12-fold increase in nesting habitat. Quail habitat use actually shifted from naturally vegetated areas to soybean plants. Furthermore, researchers observed that 3 tracked bobwhite broods were present in the field during an aerial application of insecticides (Puckett et al., 1995). Although no mortality effects were observed as a result of the exposure, it provided clear evidence that quail use agricultural habitat and are present during foliar pesticide applications.

A follow-up study more closely investigated the extent to which quail broods use agricultural fields. Researchers compared use rate in fields with and without vegetated borders, and they found that both field types were used extensively during foliar pesticide applications, but usage rate doubled in plots surrounded by natural vegetation (Palmer et al., 1998). Chicks were subject to dermal, oral, and inhalation exposure to several pesticide types, including thiodicarb, methomyl, and methyl parathion (Palmer et al., 1998). Other research has attempted to quantify the extent of the danger posed to birds by foliar sprays using pesticide use statistics and application rates, pesticide toxicity measurements, and area of land treated for each crop (Mineau, 2002). Based on these factors, corn and cotton are the most dangerous crop types for birds in terms of the potency and frequency of foliar application (Mineau and Whiteside, 2006). There is also

evidence to suggest that bird population declines in agricultural areas may be better explained by insecticide use than habitat loss due to agricultural expansion, which is oft cited as the primary cause of deteriorating populations (Mineau and Whiteside, 2013).

In addition to direct exposure from foliar sprays, bobwhites may also encounter imidacloprid through the ingestion of coated seeds, the effects of which have been documented both in the lab and in the field (Berny et al., 1999; Lopez-Antia et al., 2012; Prosser et al., 2006). During one laboratory experiment, researchers fed red-legged partridges wheat that was sprayed with imidacloprid insecticide at the recommended concentration (low dose) and at double that amount (high dose), to account for potential pesticide abuse (Lopez-Antia et al., 2012). They observed several sub-lethal effects in adult birds even at the low dosage, as well as reproductive dysfunctions which implied that toxicants were being transferred from the laying hens to their eggs (Lopez-Antia et al., 2012). The field equivalent of this experiment took place in France in the late 90s, when reports of dead pigeons and partridges in agricultural fields that had been treated with coated seeds prompted an investigation into the effects of imidacloprid ingestion (Berny et al., 1999). Bird carcasses were recovered and analyzed, whereupon it was discovered that their crops were full of coated seeds and their tissues contained significant concentrations of imidacloprid (Berny et al., 1999). Since bobwhite adults are largely granivorous (Brennan, 1999), coated seed ingestion is a highly plausible exposure route and, depending on the amount of seeds consumed, can potentially lead to magnification of the toxin in the animal's tissues.

One study attempted to quantify the threat that coated seeds pose to wild quail in the Rolling Plains ecoregion of Texas. Crop contents and liver tissues were analyzed for

clothianidin, imidacloprid, and thiamethoxam from scaled and bobwhite quail in over 30 counties in the area. No coated seeds were found in any of the 98 crops examined, and liver residues were above the threshold of detection in only 17% of samples, implying that neonicotinoid exposure may not be a significant factor in declining quail populations (Turaga et al., 2015). However, even though detected liver concentrations were described as “very low,” there were multiple detections which were several times higher than the quantitation limit. The limit for imidacloprid, for example, was 3.49 ng/g, and detected concentrations were as high as 59.05 and 62.29 ng/g (Turaga et al., 2015). The exact usage rate of neonicotinoids in the 30 counties studied in this case is also unknown, as this was determined by surveying stores in the area which may not account for all possible sources of neonicotinoid insecticides and which gives no indication as to the amount actually applied by landowners (Turaga et al., 2015). Although this work attempts to answer important questions, it does not fully elucidate the extent to which quail are exposed to neonicotinoids in the field.

Direct Mortality Effects of Pesticides

Numerous studies have examined how pesticide exposure affects the survival rate of various bird species. The U.S. Environmental Protection Agency’s toxicity evaluation for imidacloprid uses the LD50 of 2 week old bobwhites, which was found to be 152 mg/kg, as a benchmark for the chemical’s avian toxicity (Lateulere and Stavola, 1990). Other investigations have provided additional confirmatory evidence for this value and used it as a starting point for examining behavioral and reproductive effects at lower concentrations (Brewer et al., 1996; Lopez-Antia et al., 2012) or for gauging a pesticide’s

impact in a field realistic application scenario (Berny et al., 1999; Palmer et al., 1998; Puckett et al., 1995). However, several publications that focus on avian response to pesticide application feature a disclaimer that sub-lethal effects were not considered in the analysis (Mineau, 2002; Mineau and Whiteside, 2013; Puckett et al., 1995). When investigating the impacts of toxicants, mortality is often prioritized and sub-lethal effects fall outside the scope of study. However, if sub-lethal impacts can increase rates of predation, interfere with breeding potential, or shorten an organism's lifespan, then the detrimental effects of pesticide exposure are likely being severely underestimated by neglecting them (Vyas, 1999).

Mortality is a commonly used parameter in toxicological impact studies because it is generally easier to measure than sub-lethal effects, but it also features its own set of biases and challenges. Toxins that kill slowly make field mortality studies especially challenging, due to the lower chance of finding carcasses (Mineau, 2002; Prosser et al., 2006). A 1999 publication highlights several other difficulties. Quantifying mortalities can be daunting due the large scale and wide variety of possible treatment areas, as well as restricted access to private property. Carcass recovery is also problematic, largely due to scavenging activity. Studies that quantify these effects have reported that between 62 and 92% of placed carcasses are scavenged within 24 hours (Vyas, 1999; Wobeser and Wobeser, 1992). Small-scale mortality events are therefore likely to go undetected as scavengers are able to quickly dispose of the remains, resulting in underestimation of the true impact on wild populations (Vyas, 1999). It is evident that field mortality studies are restricted in their power to assess pesticide effects by ignoring sub-lethal influences—

which may alter an organism's physiology or behavior to ultimately impact its survival and breeding potential—as well as inherent limitations in data collection.

Behavioral Effects

There are several behavioral indicators of poisoning that appear consistently in birds regardless of the type of toxin involved. Some of the most frequently observed symptoms include ataxia (uncoordinated muscle movements), lethargy, wing droop, and reluctance to move or fly, which result in a general decrease in activity level (Avery et al., 1993; Berny et al., 1999; Buerger, et al., 1994). Insecticides that interfere with brain chemistry, such as the cholinesterase inhibiting organophosphates, have been known to cause seizures and tremors (Buerger, et al., 1994) while chemicals that cause acute intestinal distress can produce vomiting or diarrhea shortly after ingestion (Avery et al., 1993). Another familiar indicator of avian illness or distress is ptiloerection, the raising of feathers in an attempt to increase insulation and body temperature (Brewer et al., 1996). The aforementioned symptoms are not species specific and have been documented in studies examining blackbirds, partridges, pigeons and others (Berny et al., 1999; Lopez-Antia et al., 2012; Poppenga and Tawde, 2012).

Prior research has established a correlation between uncoordinated behavior and a decrease in levels of cholinesterase (ChE), the enzyme that breaks down the neurotransmitter acetylcholine so that it can be reabsorbed by neurons. When cholinesterase is inhibited, the result is overstimulation of the nervous system that can cause ataxia and tremors, and birds are often especially sensitive to these effects (Murphy et al., 1968; Walker, 1983). Researchers in one study observed a 23–35%

decrease in ChE in surviving northern bobwhites following an application of methyl parathion, with ChE depression in non-survivors as severe as 80%. Although ChE activity recovered to 70% of control levels following a 2 week rest period, symptoms during the period of intoxication (ataxia, lethargy, tremors) were striking (Buerger, et al., 1994).

There is also evidence to suggest that behavioral response to pesticide exposure may vary between laboratory and wild birds, as seen in a study investigating the mortality and sub-lethal effects of the organophosphate insecticide Terbufos on bobwhite quail (Brewer et al., 1996). After establishing LD50 concentrations using lab-reared birds, both lab and wild sample populations were dosed with pesticides through oral intubation and observed. Surprisingly, mortality in wild birds was not significantly higher than rates predicted by the lab studies, despite wild bobwhites having to contend with environmental variables and predation. All lab birds exhibited ataxia, ptiloerection and diarrhea. However, wild birds showed no difference in their activity levels, meaning they did not undergo long periods of lethargy (Brewer et al., 1996). Although researchers ultimately could not conclude whether wild birds exhibited abnormal behavior, in this case there seemed to be no significant impacts on their survival.

Other studies, however, have reached different conclusions. Although behavioral effects may not be directly lethal to birds, there is evidence that they can increase the likelihood of predation. Buerger et al. (1994) asserted that the sub-lethal effects of ChE depression they observed would likely make easy prey of affected birds. Other research documenting that the sub-lethal effects of lead poisoning, which inhibit the functioning of the circulatory, renal, and nervous systems, make birds more susceptible to sickness and predation while also interfering with reproduction (Fisher et al., 2006). This phenomenon

was examined directly when house sparrows were exposed to field realistic doses of “Rid a Bird” solution (active ingredient: fenthion) and then presented to American kestrels for predation (Hunt et al., 1992). The treated sparrow was the one captured In 12 of 15 total successful hunts. Five of the captured contaminated sparrows displayed abnormal behavior such as irregular preening, salivation, picking at feet, difficulty perching, and ataxia/reluctance to fly (Hunt et al., 1992). Poisoning may increase the likelihood that a prey animal is captured by making it more conspicuous to predators, by diminishing its ability to avoid/escape the threat, and by causing the afflicted animal to separate itself from the group.

Reproductive Abnormalities

Avian reproduction may be affected by insecticides in a number of ways. Changes in the quality and number of eggs laid have been well documented in a number of insecticides, the most infamous of which being the organochlorine pesticide DDT which resulted in severe eggshell thinning in birds of prey (Cooke, 1971; Fry, 1995; Jagannath et al., 2008). Similar effects have been observed due to methyl parathion ingestion. Laying hens that were treated with pesticide-contaminated food had decreased egg production within 3 days of dosing, eggshell thinning occurring after only 24 hours, and dose related reductions in both hen and egg mass (Bennett and Bennett, 1990). Reduced feeding by hens rather than direct toxic effects may have caused these particular results. Other literature reports that imidacloprid and clothianidin have directly contributed to eggshell thinning in birds, as well as reducing embryo size, hatching success, and chick survival (Gibbons et al., 2014).

The eggshell itself can serve as a visual indicator of toxin exposure. One study examined how shell pigmentation changes in response to contamination. It was found that specks of color referred to as protoporphyrin speckling are more numerous on low-calcium (thinner) areas of the eggshell, and in some species, vibrancy of color indicates the health of the female (Jagannath et al., 2008). Researchers verified this correlation through analysis of Eurasian sparrowhawk eggs for concentrations of DDE, a metabolite of DDT. Eggs containing greater amounts of DDE featured more speckling (corresponding to thinner shell areas) and increasing green hue intensity (Jagannath et al., 2008). A similar trend has been noted in the poultry industry, where eggs from hens exposed to a toxicant display altered pigmentation (Odabaşi et al., 2006). There is no guarantee that neonicotinoids will produce similar visual indicators of exposure, but it is another potential metric for evaluating the pesticides' effects.

In another study investigating the reproductive impacts of neonicotinoids, researchers performed a statistical analysis concerning the effects of thiamethoxam, thiacloprid, and acetamiprid on 3 different bird species—northern bobwhites, mallard ducks (*Anas platyrhynchos*), and Japanese quail (*Coturnix japonica*) (Fernandez -Perea et al., 2009). They found that reproductive response to a given pesticide is highly species dependent. Bobwhite eggs had a higher tolerance to pesticide solution exposure than mallards, which is attributed to differences in eggshell thickness (the thick cuticle in bobwhite eggs provided added protection). However, bobwhites were more sensitive in cases of ingestion by the laying hen because mallards are known to regurgitate after force-feeding. Significant differences were found between acetamiprid treatments and other neonicotinoids for "eggs laid" and "embryo survival," which may be related to

acetamiprid's rapid metabolism. There were no differences among neonicotinoids for eggs set, fertilized and hatched, nor for 14 day survivorship of chicks or eggshell thickness (Fernandez -Perea et al., 2009).

Reproductive impediments are not limited to eggs, however; there is also evidence to suggest that clothianidin may diminish the virility of male quail and the laying ability of hens. When males were treated with pesticide and then bred with untreated females, there was a decrease in embryonic length according to dosage, and at the highest concentration, several eggs did not develop. Additionally, some of the adult birds in the highest dose group displayed overt behavioral evidence of poisoning, such as ruffled feathers and convulsions. No differences in egg weights and fertilization rates were observed among treatments (Tokumoto et al., 2013). In a follow-up study the following year, treated males once again produced smaller embryos that were less likely to fully develop, and treated females produced fewer eggs, smaller embryos, and deformations in their ovaries (Hoshi et al., 2014). It is worth considering that laboratory toxicity studies which fail to account for all field-relevant aspects of reproduction—behavioral changes, multiple routes of exposure, impact on subsequent breeding attempts in a season, etc.—may be underestimating the true impact (Bennett and Etterson, 2006), and any such reproductive difficulties could affect bobwhite nesting success and recruitment.

Pesticide impacts on embryos are an additional concern and will depend upon the particular route of exposure. One study compared two exposure routes—transfer from dosed hens and direct injection into the egg—while investigating DDT/DDE uptake by quail embryos (Cooke, 1971). Uptake of the chemical was slower in injected eggs, though those embryos also exhibited slower development. These affects are partially attributed

to the influence of the injection solvent or vehicle (Cooke, 1971). Research also indicates that the injection site and method are critical in determining the chick's response, and that chemicals injected harmlessly into the yolk may be toxic in the air cell, and vice versa (Gebhardt and Van Logten, 1968).

Insecticides may have significant reproductive impacts by altering the developmental rate or physiology of the chicks. Chemical exposure has been shown to reduce brain cholinesterase activity and body mass in chicks as well as adults (Palmer et al., 1998). Effects on developing embryos are often more profound than those on adult birds and may occur at doses below the established LD50, even when lethal dose for adults is fairly high (Dabbert et al., 1997). Other documented effects on embryos include increased mortality/lower hatchability, failure to grow post-hatch, skeletal and beak defects, and hormone disruption resulting in abnormalities of the reproductive system (Fry, 1995; Gibbons et al., 2014). Heart deformities and swelling in tissues are also ailments observed in embryos exposed to toxicants (Fry, 1995).

Other Sub-Lethal Effects

Neonicotinoids, specifically imidacloprid and clothianidin, have been shown to cause the same incoordination and lethargy observed in other pesticide tests in addition to causing destruction of DNA and suppression of immune response in sparrows, Japanese quail, and partridges. These effects were observed at concentrations considerably lower than those that are known to cause mortality, in some cases a full order of magnitude below lethal concentrations—a rare occurrence in other pesticide families (Gibbons et al., 2014). Recent work has also demonstrated that sub-lethal

nutritional stress—potentially augmented by toxicant exposure—during periods of growth and development can alter organ mass and immunocompetence in adult birds (Kriengwatana et al., 2013). There is evidence implying that these and other sub-lethal impacts of neonicotinoids are having a measurable effect on avian populations, as a recent publication reveals that imidacloprid residues in the environment are the best correlate of bird population declines in the Netherlands, even when considering agricultural intensification, land use changes, and applications of other chemicals (Hallmann et al., 2014).

Other research has documented insecticide effects on food sources, habitat availability, and predator/prey relationships which may have indirect impacts on species of interest. Research on the nesting and brood rearing habits of Yellowhammers and Skylarks reveals that insecticides threaten these species indirectly by reducing invertebrate food sources for their chicks (Boatman et al., 2004; Gross, 2014). Bobwhite quail chicks are also highly dependent on insects as an early protein source (Brennan, 1999). Another concern is that pesticides (herbicides, especially) can reduce habitat for invertebrate prey items and the birds themselves, thereby affecting avian populations independently of direct toxic effects (Boatman et al., 2004). There should also be consideration for secondary poisoning in predators that feed on affected prey, as was the case in Hunt et al. (1992) where kestrels targeted pesticide exposed sparrows. Predators may be impacted both by a reduction in food source populations and by bioaccumulation of toxins in their own bodies due to preferential feeding on intoxicated and impaired prey (Walker, 2003).

Genetic Response to Pesticide Exposure

Previous work has demonstrated that chemical exposure can alter physiology at the level of gene expression and transcription. In quail, the organochlorine pesticide dieldrin has been shown to decrease mRNA expression of tryptophan hydroxylase (an enzyme involved in serotonin synthesis) in the brain stem, as well as increase mRNA expression of certain key proteins (e.g. terminal oxidase enzymes) in the liver (Kamata et al., 2010). During a 2013 study in which adult quail were treated with chronic oral doses of clothianidin, increased DNA fragmentation in the liver was one of several resultant irregularities (Tokumoto et al., 2013). There have also been documented epigenetic effects of endocrine disruptor pesticides altering DNA methylation patterns in rats, resulting in malformed reproductive organs in males and females exposed during gestation (Collotta et al., 2013). The effects of contaminants may be especially pronounced when exposure occurs during development. For example, fetal exposure to nicotine through the mother's bloodstream or breast milk has been shown to increase the number of nAChRs created during the formation of the central nervous system, which can play a role in a number of diseases and disorders later in life (Gotti and Clementi, 2004). For neonicotinoids specifically, one study investigated how mussel (*Mytilus galloprovincialis*) gene expression changed in response to imidacloprid and thiacloprid and found that there were 97 and 80 genes, respectively, which were differently expressed in exposed groups (Dondero et al., 2010). Most of these variations were not shared between the two neonicotinoid types, suggesting that different varieties can affect gene expression in unique ways.

If pesticides exposure can result in heritable DNA changes, then an evolved tolerance to the chemical is also a real possibility. Researchers in a 2011 study examined gene transcription to determine how separate killifish populations were able to withstand toxic levels of dioxins, PCBs and metals that should have proved lethal to embryos and fry. Genetic analysis of the embryos revealed that different killifish populations had independently evolved a mechanism to resist the effects of dioxin-like pesticides, made possible by a genotype that was present in ancestral populations (Whitehead et al., 2011). Evolved tolerance to neonicotinoids has also been documented and is becoming an impediment to the control of pest species. For example, two biotypes of the hemipteran *Bemisia tabaci* have developed a strong resistance to imidacloprid which is attributed to over-expression of the gene CYP6CCM1 and increased activity of P450 detoxifying enzymes (Karunker et al., 2008). Whether quail have the capacity for pesticide tolerance will also depend on the pre-existence of genotypes that grant physiological resistance to the chemical, possibly by effecting more efficient metabolization, better sequestration and excretion mechanisms, or fewer receptors to respond to the toxin's effects.

Research Objectives

Prior investigations have documented organ mass changes in embryos exposed to various toxins. Exposure to the dioxin compound TCDD, for example, has been known to decrease the mass of embryonic zebrafish hearts by reducing the number of cardiomyocytes (Antkiewicz et al., 2005). The same compound, when administered to chicken embryos, produced larger hearts and smaller livers, with effects varying during different periods of exposure (Bruggeman et al., 2003). In another case, mouse embryos

subjected to bisphenol A exhibited decreased mass of the brain, testis and kidneys (Kabuto et al., 2004). One objective of this research, therefore, was to determine if neonicotinoids similarly impact embryonic organs by examining the mass of the heart, liver, lungs and kidneys.

Other documented effects of toxicants on embryos include higher mortality rates and frequencies of anatomical malformation. For example, thalidomide has been known to produce severe limb defects in human fetuses (Kim and Scialli, 2011), and there is evidence connecting neonicotinoid exposure with deformities of the skeleton and reproductive organs in chicken embryos (Gawade et al., 2013; Seifert and Stollberg, 2005). Investigations examining these effects also frequently observe increased embryo mortality in exposed groups (Gibbons et al., 2014; Hoffman and Albers, 1984; McEvoy et al., 2001). Based on this information, the second objective of this research was to determine if mortality and prevalence of anatomical deformities are also higher in bobwhite quail embryos exposed to neonicotinoids.

Finally, embryos present unique opportunities and challenges in toxicology testing because they are in the process of forming their bodily structures. Genes are activated and tissues are constructed at different points throughout the incubation process, and so the timing of toxicant exposure is especially important in determining its effects. Previous work has investigated how hypoxic conditions impact the development of organs and organ systems during various stages of development (Burggren and Reyna, 2011; Chan and Burggren, 2005) and how lead exposure affects rat embryos during both early and late gestational periods (Bunn et al., 2001). Numerous types of feed and forage toxins have also been known to disrupt fetal growth in calves in

different ways (e.g. skeletal abnormalities, reduced organ functionality) depending on when exposure occurs during the pregnancy (McEvoy et al., 2001). Based on these observations, the third research objective in investigating the effects of neonicotinoids on bobwhite quail embryos was to identify critical windows of development when changes in organ mass and anatomy are more likely to occur.

Materials and Methods

Egg storage and incubation

A total of 650 bobwhite eggs were obtained from Quail Ranch of Oklahoma (Wardville, OK) and Strickland Game Birds (Pooler, GA) in sets of 100–200 eggs per group. Shipments were examined immediately upon arrival, and cracked eggs were discarded while those remaining were transferred to plastic quail egg racks and oriented blunt-end up. Racks were stored in the laboratory at room temperature (25–28 °C) prior to incubation. All eggs were incubated within 2 weeks of arrival to avoid significant loss of hatchability (Reyna, 2010).

Incubation occurred in two incubators (GQF; Savannah, GA). One incubator was designated exclusively for control treatments while the other was for neonicotinoid dosed eggs. Both incubators were maintained at a temperature of 38 °C and 66% relative humidity for the duration of all experiments, and both were programmed to turn the eggs automatically once per hour. Incubating eggs were candled every 3 days to verify development; those that failed to initiate or ceased development over the course of incubation were noted and discarded.

Eggs were set and processed after 19 days of development over the course of 3 experiments. Experiment 1 (n = 200) and experiment 2 (n = 200) were primarily focused on quantifying changes in organ mass during embryonic development with imidacloprid exposure at multiple concentrations, while experiment 3 (n = 250) was more focused on imidacloprid effects on heart mass at specific concentrations and developmental periods.

Experimental treatment

Neonicotinoid treatments were administered via injection directly into the air cell of each egg. Eggs were weighed, set, and then processed after 19 days of development over the course of 3 experiments. Experiment 1 (n = 200) and experiment 2 (n = 200) were primarily focused on quantifying changes in organ mass during embryonic development with imidacloprid exposure at multiple concentrations, while experiment 3 (n = 250) was more focused on imidacloprid effects on heart mass at specific concentrations and time periods during development. This research was approved by the University of North Texas Institutional Animal Care and Use Committee (IACUC) protocol number 1205-08.

Treatment solutions consisted of the imidacloprid based insecticide Merit® 75 WSP (Bayer Corporation) dissolved in a liquid vehicle. Two different vehicles were used throughout the investigation: experiments 1 and 3 included a 0.9% saline solution, while eggs in experiment 2 were subjected to dimethyl sulfoxide (DMSO). The DMSO vehicle was used to test the effects of increased Merit solubility. Dosing solutions were prepared just prior to injection and were blended at 300 RPM for 60 seconds using a laboratory mixer. To administer the solution, an 18 gauge needle was used to puncture the shell and

outer membrane in the center of the blunt end of each egg, then a 100 µL syringe was inserted just past the bevel to deliver the test solution (approximately 20 µL). Following injection, the puncture was sealed using silicone gel, and eggs were returned to the incubator.

There were 5 treatment concentrations based on the mass of the egg at time of dosing. These included doses of 0, 10, 50, 100, and 150 mg Merit per kg egg mass. The 0 mg/kg solution was a sham consisting only of the dosing vehicle without any Merit, while the 150 mg/kg treatment was 2 mg/kg below the LD50 value for bobwhites (Lateulere and Stavola, 1990). In addition to varying in concentration, treatments were also administered at different periods of development. Dosing occurred after 0, 3, 6, 9, or 12 days of incubation. These will be referred to as incubation days, with day 0 representing exposure just prior to the start of incubation. Experiments 1 and 2 examined all treatment combinations, while 3 focused on sham and 150 mg/kg dose administered at day 0, 6 and 12. In all cases, at least 8 eggs were set for each treatment, and each egg was subject to only one dose and a single instance of exposure (i.e. only one injection).

Dissection and processing

All embryos were removed and dissected after 19 days of incubation, approximately 80% of the 23 total days required for bobwhite chicks to hatch (Klimstra and Roseberry, 1975). Prior to dissection, embryos were euthanized using isoflurane anesthetic. Upon removal from the egg, embryos were weighed and beak and tarsus length were recorded for staging purposes as previously described (Hendrickx and Hanzlik, 1965). Embryos were opened via a longitudinal incision down the center of the

body, and the heart was removed by cutting around the pericardium and severing the aortic arch at its base. The liver was then extracted, with the gall bladder separated and discarded. Both lungs and both kidneys were removed and in each case were weighed as a pair. The wet masses of the organs were recorded; then they were transferred to 1.5 mL microcentrifuge tubes, submerged in RNA later solution (Ambion Inc.; Austin, TX), and stored in a -18 °C freezer to preserve tissues for future use. During dissection, any deformities in the embryos or their organs were noted.

Statistical analysis

Organs were compared between treatments by calculating the ratio of each organ mass to the mass of the embryo from which it was removed. These ratio values were then subjected to arcsine transformation ($\sin^{-1}\sqrt{(x)}$) and used in a series of 1-way ANOVAs and t tests which compared average masses of heart, liver, lungs and kidneys among all treatments. All statistical tests were performed at $\alpha = 0.05$. When significant differences were detected, pairwise multiple comparisons were performed using either the Holm-Šídák approach or Dunn's multiple comparison test to determine the specific treatment combinations that were significant. Embryonic mass was evaluated using almost identical methods, except no arcsine transformation was necessary because raw mass values were used instead of converted ratios.

Beak lengths and tarsus lengths were measured and analyzed using established staging criteria to determine each embryo's stage of development at the time of dissection. If beak staging did not agree with tarsus staging for an individual embryo, then the mean staging value was used in subsequent calculations. Bobwhite embryos are

expected to reach stage 38 of development after 19 days of incubation (Hendrickx and Hanzlik, 1965); embryos in each treatment that lagged behind this expected value by more than 24 hours (less than stage 37) were counted and compared among day and dose treatment groups in each experiment.

Anatomical deformities were placed into categories based on affected body part. These included malformations of the beak, feet, legs and eyes, as well as severe underdevelopment (i.e. lacking significant developmental milestones, such as feathers and leg scales, which are expected at day 19). Embryos that exhibited multiple deformities were counted in all applicable categories. The percent of total deformities occurring at each day and dose was calculated, as well as the percent of each specific deformity type. These were compared between treatments to determine the dosing conditions correlated with greater prevalence of malformation.

Cox proportional hazard models were constructed to determine how day and dose affected survival as covariates. Embryos that continued to develop up to the date of processing were identified as survivors and listed as “censored”, while those that were removed during incubation due to having ceased development (as determined by candling) were counted as mortality events. Some embryos were also revealed to be deceased upon opening the egg and were labeled mortalities. Additionally, mortality rate was reported in terms of percentage of survivors and deaths in each treatment.

Results

Organ mass differences between treatments

Embryonic heart mass ratios varied with imidacloprid dose and timing of application. In day 0 treatments for experiment 1, the ratios of heart mass to embryonic mass (mg/g) in the sham (7.1 ± 0.3), 10 (7.1 ± 0.4) and 50 (6.7 ± 0.2) treatments were significantly lower than that at the 150 mg/kg dose (9.1 ± 0.5; Table 1). There were no differences between heart ratios among the 10, 50 or 100 mg/kg dosing groups at day 0 (Fig. 1A). Heart ratios were also greater at higher dosing concentrations in day 9 treatments (Fig. 1B), with the ratio in the sham dose group (6.7 ± 0.1) being significantly smaller than in the 150 group (7.8 ± 0.4; Table 1). In experiment 3, heart mass ratios were marginally significant between sham and 150 treatments at day 6 ($p = 0.053$), again correlating positively with dose.

Variation also occurred in lung mass ratios in experiment 1. Differences were between the 10 vs 150 and 50 vs 150 groups at day 0 (Table 1), and in these cases, the ratio (g/g) of lung to embryonic mass in the 150 treatment (0.0059) was significantly smaller than in both the 10 (0.0082) and 50 (0.0080) dose groups (Fig. 2A). Sham and 150 doses were not statistically different at day 0 ($p = 0.07$). Differences in lung ratios were also observed at day 12 between the sham vs 150 and 50 vs 150 treatment groups (Table 1). Embryos in these treatments featured significantly greater lung/embryo mass ratios at higher doses (Fig. 2B), contrary to the effects observed at day 0.

Liver and kidney mass ratios differed among dosing concentrations at incubation day 6 and 12. Complications in experiment 1 and increased mortality in experiment 2 only allowed for comparisons between sham and 10 mg/kg doses for day 6. Despite limitations, day 6 featured variation in both liver ($p = 0.018$) and kidney mass ratios ($p = 0.036$) in experiments 1 and 2, respectively. Day 6 liver mass ratios were positively

correlated with imidacloprid dose (Fig. 3A), a trend which also occurred in the liver at day 12 (Fig. 3B). Differences in day 12 organs were observed only in experiment 1. Kidney ratios in the day 6 treatment groups showed a similar trend of greater relative kidney mass among imidacloprid dosed embryos relative to sham specimens (Fig. 4). Although there were no differences in liver or kidneys at day 9, liver ratios in this case were fairly close to significance ($p < 0.066$).

Embryonic mass and developmental rate

There were differences in embryonic mass correlating to imidacloprid exposure at nearly every window of development. Differences occurred in experiment 1 at day 0, 3, and 9 (Table 1). Day 0 differences were between the sham and the 10, 50 and 150 mg/kg doses, with significantly lower embryonic mass averages occurring in neonicotinoid exposed embryos (Fig. 5A). Day 3 treatments exhibited differences between the sham vs 10 and 10 vs 50 dose groups (Fig. 5B), and mean embryonic mass at the 10 mg/kg dose (3.45 ± 0.2 g) was significantly smaller than at either the sham (4.69 ± 0.1 g) or 50 (4.62 ± 0.2 g) treatments. At day 9, differences were attributed to the sham vs 150 and 50 vs 150 treatment comparisons (Fig. 5C), with average mass among 150 dose embryos (3.96 ± 0.2 g) 12% lower than sham specimens (4.50 ± 0.1 g; Table 1). Experiment 3 also featured embryonic mass variation at day 0 and 12, in both cases with highly significant p values < 0.001 and a trend of lower mass at higher doses. There was an 18% decrease mass between sham and 150 embryos at day 0 and a 25% decrease at day 12, constituting a difference of more than a gram between sham and 150 averages in the latter case.

Although embryonic mass varied in response to neonicotinoid dosing, stage of development exhibited no significant variation among treatments and in most cases matched the expected value of stage 38 for quail embryos at day 19 of incubation (Hendrickx and Hanzlik, 1965).

Anatomical deformities

Several deformity types were observed over the course of the investigation, most of which occurred during experiment 2. Among the experiment 2 embryos, every category of deformity—e.g. “beak,” “legs,” “eyes,” and “underdeveloped”—was represented at least once (Fig. 6). Forty-one percent of subjects exhibited some type of malformation, and one individual counted in two categories (beak and legs). It should be noted that malformations were not limited exclusively to neonicotinoid treated embryos, and sham subjects accounted for 6 of the total cases including 1 beak deformity, 3 legs, and 2 underdeveloped specimens. Severe underdevelopment was the most common anomaly (n = 17), which was present in 22% of experiment 2 embryos and accounted for about half of all malformations, followed by leg (n = 7) and beak (n = 5) defects.

A few specific treatments in experiment 2 also stood out in terms of deformity frequency. Of the 31 total cases, 26% were observed in the day 12/dose 150 treatment group, followed by 16% in day 12/dose 100 and 10% in both day 6/dose 100 and day 3/dose 50. When all doses were considered together, 45% of deformities occurred at day 12, though these belonged exclusively to the “Underdeveloped” category, followed by day 6 with 29% of the total. When all days were combined and dosing concentration was

examined, the 100 and 150 mg/kg treatments had similarly high deformity rates at 29% each, accounting together for more than half (58%) of all cases (Fig. 7A and 7B).

Mortality

Cox proportional hazard models illustrated the effects of incubation day and dosage concentration on survival of the embryos. The model for experiment 1 indicated that hazard rate varied significantly among doses (P = 0.006) but not among IDs (P = 0.490). When all incubation days were compared, day 0 exposure had the lowest percentage of survival (66.7% survival; 33.3% mortality) while days 9 and 12 had the highest (96.9% and 90.6% survival, respectively). Among the various dosing concentrations, sham solution embryos predictably had the highest survival rate at 92.5%. Dose 10 exhibited an unusually high 43.8% mortality, followed by the dose 100 treatment (25% mortality) and dose 150 treatment (13% mortality).

Experiment 2 had considerably lower overall rates of survival than those observed in experiment 1. When all experiment 2 embryos were considered together, mortality rate was 62% in contrast to a 19% overall mortality rate during the first experiment. Despite increased rates of mortality, there were still differences among specific days and doses. Day 3 and 9 had the fewest survivors in this case (25% and 18% respectively); day 12 had the most with a survival rate of 60%. Mortality and dose were positively correlated, with more deaths at higher concentrations and the greatest mortality rate (90%) occurring in the 150 mg/kg treatment. Hazard rate was significantly correlated with both day and dose as indicated by the Cox model, with p values < 0.001.

Experiment 3 included a “no dose” group which was not subject to any kind of injection in addition to the sham and 150 treatments. The overall survival rate fell between that of experiment 1 and 2 with 68.1% survival and 31.9% mortality. Cox proportional hazard analysis suggested that dose was a predictor of hazard rate ($P = 0.042$), but day had no significant connection ($P = 0.996$). When survival percentage was examined in terms of day and dose, mortality rates at day 0 and 6 were approximately equal (25.9% and 26.4%, respectively) while day 12 featured an increased mortality rate of 54%. Dosing concentration exhibited the same trend seen in experiment 2, as mortality correlated positively with dose and the 150 treatment had the greatest percent mortality (41.1%).

Discussion

This investigation lends support to the claim that embryonic exposure to imidacloprid administered via injection into the air cell affects heart and lung mass, body mass, and anatomical integrity. These effects are mediated by both the period of application and the dose of imidacloprid administered. Previous research has established that several types of toxicants can alter the size of embryos (Bruggeman et al., 2003; Gibbons et al., 2014; Tokumoto et al., 2013) as well as organ systems and anatomy (Bruggeman et al., 2003; Fry, 1995; Gawade et al., 2013). Although the impacts of pesticides from previous decades have been thoroughly studied, the physiological effects of the newer and more ubiquitous neonicotinoid insecticides are only beginning to be understood, especially in relation to non-target vertebrate species like bobwhite quail that are likely to encounter such chemicals in their environment.

Organ mass variation has been previously documented in studies concerning different species and toxicants. Bruggeman et al. (2003) examined the effects of TCDD, a dioxin compound, on chicken embryo development and found that embryos exposed to a moderate dose at 4 days of incubation had relatively larger hearts, while embryos exposed to a high dose at 8 days of incubation produced smaller livers and larger hearts than in other treatments. Similarly, this investigation showed that imidacloprid exposure correlated with larger hearts at incubation day 0 and 9, larger livers and kidneys at day 6, and larger livers at day 12, with the highest dosing concentrations having the greatest impact. Lung masses were especially interesting in that they were smaller at day 0, but larger at day 12, suggesting that the timing of exposure is as crucial as dosage in determining imidacloprid's effects. Previous work has established a correlation between heart mass and maximum cardiac output (Bishop, 1997), implying that organ size and functionality may be related. The cause for an increase in organ mass is difficult to discern from wet mass alone, however, and may be due to more functional, healthy tissue or to non-functional scar tissue. In any case, variation is evidence of imidacloprid interfering with organ growth and altering developmental pathways.

Embryonic mass also responded to imidacloprid treatment, with smaller embryos occurring at higher dosing concentrations in all incubation days. Other work has documented a positive correlation between embryonic mass and hatching success and survival rates (Gibbons et al., 2014; Tokumoto et al., 2013) as well as failure to grow post-hatch with decreasing embryonic mass (Fry, 1995). The hatching and early post-hatch periods are sensitive and dangerous times for birds, as egress from the egg requires time and substantial energy expenditure that leave new hatchlings exhausted and vulnerable

to predation. A “small” chick, such as the dose 150 individuals that were a full 25% smaller than their sham counterparts at day 12, is disadvantaged in competition with larger siblings for space and resources and may have greater difficulty surviving during the hatching and nesting process. Despite mass differences, dosed embryos continued to achieve developmental milestones established by staging criteria, implying that the discrepancies were not due to beak or tarsus length. Variation is more likely attributable to organ or muscle mass differences.

Imidacloprid exposure also appeared to increase the frequency of deformed embryos. Most deformity cases occurred during experiment 2 when the dosing vehicle was switched from a saline solution to dimethyl sulfoxide, implying that the DMSO itself may have been a factor. DMSO has frequently been used as a dosing vehicle in embryonic toxicology studies, with some reporting it to be innocuous and others suggesting that it may exert its own toxic effects. Winter et al. injected 5 microliters of DMSO into zebra finch eggs averaging 1 gram in mass and found no impact on embryonic development or hatchability, but they did note significantly lower body mass in male chicks at 21 and 90 days post-hatch (Winter et al., 2013). Scialli et al. used DMSO to evaluate the effects of taxol on chicken embryos and postulated that the vehicle was toxic when injected into the air cell, though they did not maintain sham and non-manipulated control treatments (Scialli et al., 1994). In contrast, O’Brien et al. and Heinz et al. used DMSO vehicles to administer perfluoroalkyl compounds and methylmercury, respectively, into chicken egg air cells and observed no differences between sham and untreated control groups (Heinz et al., 2006; O’Brien et al., 2009). Other work implies that DMSO

may increase or decrease the toxicity (as indicated by LD50) of the compounds dissolved in it (Rosen et al., 1965).

Despite potential interference from the dosing vehicle, it is important to remember that all embryos in the experiment 2 treatment groups were subject to DMSO exposure and that there is still variation in deformity frequency between treatments. The majority of anatomical malformations occurred in the two highest neonicotinoid dose groups. Other toxicological studies have established a similar link between chemical exposure and defects in embryos. DDT has long been known to cause skeletal and beak abnormalities in birds (Fry, 1995) while imidacloprid and clothianidin have produced testicular deformities as well as malformed ribs, vertebrae, and phalanges in vertebrate species (Gawade et al., 2013; Gibbons et al., 2014). Additionally, imidacloprid's interference with neuromuscular nAChRs has been cited as a likely cause of curvature of the joints in embryos (Seifert and Stollberg, 2005). These teratogenic effects can affect survivability of quail embryos and chicks in substantial ways. A quail with a malformation of the beak, for example, may have greater difficulty pipping the eggshell and feeding post-hatch. One with deformed joints or feet would struggle to keep up with its mother and siblings, a challenge faced by all precocial chicks shortly after hatching. Even relatively minor malformations could interfere with essential predator avoidance.

Likely as a result of interactions among multiple factors, overall probability of survival was lower at higher dosing concentrations. In all cases, dose concentration was a significant factor affecting hazard rate, and both dose and day were critical covariates in experiment 2. If imidacloprid exposure, as previously discussed, was interfering with the biochemistry of the embryo, changing gene transcription rates or damaging DNA, and

subsequently altering organ mass and anatomy, then overall survival rates would also be affected. The fact that survival declined well below the highest dosage, which was just below the established LD50, is especially interesting, as there were significant differences even among the lowest treatment concentrations such as the sham and 10 mg/kg dose. One possible explanation for this is that embryos are more sensitive to imidacloprid's effects than the adult birds used in the Environmental Protection Agency's LD50 tests, a phenomenon which has been observed in studies examining other species and toxicants. Previous work has shown that larval zebra fish (*Danio rerio*) exhibit changes in development, behavior, and neural physiology in response to neurotoxin exposure, and these alterations occur at levels which are not detrimental to adults (Bretaud et al., 2004). In another study, adult mallards fed a diet containing methylmercury experienced decreased reproductive success (fewer eggs laid, fewer hatched) while their ducklings displayed altered behaviors in the form of diminished sensitivity to maternal calls and hypersensitivity to frightening stimuli (Heinz, 1979). Perhaps the most infamous example is that of thalidomide, a drug previously used to treat morning sickness in pregnant women which caused severe limb defects in fetuses (Kim and Scialli, 2011). In each case, doses which produced minimal adverse effects in adults had drastically more serious consequences for developing organisms.

When the effects on organ mass, embryonic mass, and deformity frequency are considered together, there appear to be "critical windows" of development when bobwhite embryos are relatively more sensitive to imidacloprid exposure. Previous research concerning critical windows suggests that there are periods of development when specific body structures and systems are especially susceptible to the effects of stimuli as heat

stress, hypoxia, and toxicant exposure. Changes during these critical periods can subsequently alter developmental trajectory and phenotype. (Burggren and Reyna, 2011; Drake et al., 2006). In this case, the pre-incubation exposure (day 0) was an important period for affecting heart and lung mass. This might be surprising given that there are no bodily structures present at this time for the chemical to influence, but there are stem cells present. Damage to stem cells could impact any aspect of development, and with the heart being one of the earliest structures to form, its growth would likely be affected by chemical exposure at the beginning of the incubation process. All of the windows investigated also produced smaller embryos, implying that embryonic mass may be a sensitive parameter throughout development. When examining deformities, day 12—a point in embryonic growth when limbs and phalanges are formed but feathers are still absent—emerged as a period of concern in producing underdeveloped embryos, but did not produce deformed beaks or legs. If other malformation types are considered independently, then day 6—a period when a quail embryo has two eyes but only buds for limbs—features a higher frequency of beak and leg abnormalities. These observations suggest that neonicotinoid exposure has the most profound effects on anatomical structures yet to be formed, and that exposure just prior to the development of a structure may increase its chance of malformation.

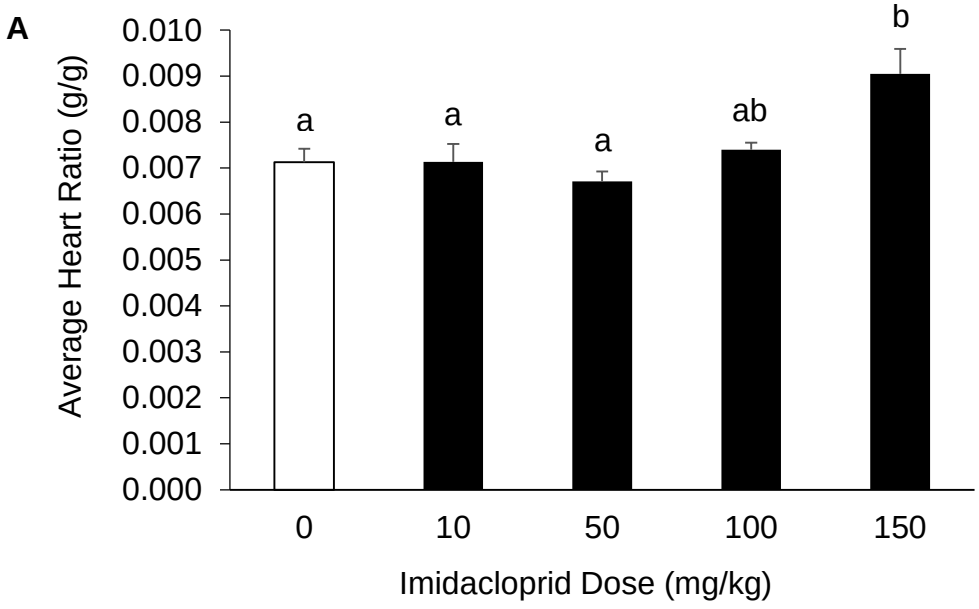
This investigation has demonstrated that imidacloprid exposure affects the physiology and development of bobwhite quail embryos. The doses tested here (0, 10, 50, 100, and 150 mg/kg) cover a range that falls just below the accepted LD50 of 152 mg/kg (Lateulere and Stavola, 1990), and significant impacts on liver, kidney, and embryonic mass were observed even at the relatively low 10 and 50 mg/kg

concentrations. Research has attempted to gauge the consumption of neonicotinoid-coated seeds by adult wild quail and measure concentrations of the chemical in their tissues. It was concluded that the pesticide poses little threat to scaled and bobwhite quail in the field based on an absence of coated seeds in the crop and low detections in liver samples (Turaga et al., 2015), but the study did not address the possibility that chicks and developing embryos may be disproportionately affected by exposure at concentrations below toxic levels for adults. Determining field relevant rates of transfer to eggs from either hen or environment therefore remains a pertinent subject for future studies. Though direct application rates of neonicotinoids are purportedly low (Elbert et al., 2008), the chemicals are known to be environmentally persistent and to accumulate in soil and water over time (Henk and Sanchez-Bayo, 2011), meaning that the actual rate of exposure to wildlife and the detrimental effects of that exposure are likely being underestimated. This problem is compounded by the fact that most prior studies fail to account for the sub-lethal, developmental impacts observed in these experiments which can impact survival.

Table 1. Mass of the embryo, heart, liver, lungs and kidneys, and the average ratio of each organ to embryonic mass in all treatment groups after 19 days of incubation. Treatment groups are denoted by the day of exposure and dosing concentration (milligrams imidacloprid per kilogram egg mass). Dotted lines separate Incubation Days, and differing superscripted letters indicate significant variation. Data are presented as the mean value ± SE.

Day	Dose (mg/kg)	N	Embryonic Mass (g)	Heart (mg)	Heart/ embryo (mg/g)	Liver (mg)	Liver/ embryo (mg/g)	Lungs (mg)	Lungs/ Embryo (mg/g)	Kidneys (mg)	Kidneys/ embryo (mg/g)
0	SHAM	6	4.4±0.1^{a}	31.5±1.1	7.1±0.3^{a}	103.3±5.7	24.9±1.4^{a}	34.2±1.1	8.2±0.3^{ab}	32.4±2.0	7.8±0.5^{a}
0	10	8	3.7±0.2^{b}	27.3±2.2	7.1±0.4^{a}	89.4±4.7	24.2±1.0^{a}	29.4±2.5	8.2±0.4^{a}	32.0±2.3	8.5±0.4^{a}
0	50	7	3.8±0.2^{b}	25.9±1.2	6.7±0.2^{a}	87.2±6.7	22.6±0.8^{a}	31.4±3.6	8.0±0.5^{a}	32.9±3.3	8.5±0.5^{a}
0	100	5	4.0±0.1^{ab}	29.8±0.9	7.4±0.2^{ab}	96.8±4.6	24.0±0.8^{a}	31.8±3.1	7.8±0.5^{ab}	34.8±1.1	8.6±0.2^{a}
0	150	6	3.5±0.3^{b}	31.5±3.1	9.1±0.5^{b}	74.2±8.2	21.4±1.5^{a}	21.0±3.7	5.9±0.7^{b}	25.7±4.1	7.3±0.8^{a}
3	SHAM	8	4.4±0.3^{a}	29.4±1.3	6.9±0.4^{a}	91.7±7.1	21.3±0.9^{a}	31.4±4.4	7.5±0.4^{a}	33.2±3.5	7.9±0.5^{a}
3	10	5	3.4±0.2^{b}	23.9±0.5	7.0±0.3^{a}	90.5±7.0	26.9±3.4^{a}	30.6±2.9	8.8±0.6^{a}	31.4±3.5	9.2±1.1^{a}
3	50	7	4.6±0.2^{a}	33.9±3.3	7.2±0.5^{a}	85.7±8.8	18.1±1.7^{a}	38.6±9.3	9.6±2.1^{a}	36.2±1.5	7.8±0.2^{a}
3	100	6	4.2±0.2^{ab}	28.6±2.2	6.9±0.5^{a}	86.8±5.9	21.0±1.7^{a}	31.1±3.8	7.3±0.7^{a}	32.0±2.7	7.6±0.6^{a}
3	150	5	4.1±0.2^{ab}	30.8±2.6	7.7±0.8^{a}	90.0±10.5	22.1±2.3^{a}	26.6±2.0	6.6±0.4^{a}	31.9±3.7	7.8±0.7^{a}
6	SHAM	7	4.4±0.3^{a}	29.1±1.3	7.1±0.3^{a}	88.6±7.4	20.7±1.0^{a}	29.2±3.3	7.1±0.4^{a}	35.2±5.6	7.8±0.9^{a}
6	10	5	3.5±0.5^{a}	27.5±2.0	8.5±1.0^{a}	89.6±8.9	27.0±2.4^{b}	23.2±5.3	6.3±1.1^{a}	27.3±4.3	7.7±0.4^{b}
9	SHAM	8	4.5±0.1^{a}	30.3±1.2	6.7±0.1^{a}	100.1±2.3	21.9±0.3^{a}	34.9±1.7	7.6±0.3^{a}	37.5±1.9	8.2±0.3^{a}
9	50	8	4.6±0.1^{a}	35.0±1.6	7.4±0.2^{ab}	103.2±3.3	22.5±0.6^{a}	34.9±2.2	7.6±0.4^{a}	39.5±1.7	8.6±0.4^{a}
9	100	7	4.2±0.2^{ab}	28.6±2.5	6.7±0.3^{a}	100.1±7.8	23.4±0.7^{a}	34.8±2.7	8.5±0.4^{a}	40.5±5.5	9.3±0.8^{a}
9	150	7	4.0±0.2^{b}	29.3±1.2	7.8±0.4^{b}	96.9±5.1	24.5±1.0^{a}	26.5±3.8	6.8±0.7^{a}	33.8±3.2	8.6±0.6^{a}
12	SHAM	7	4.4±0.1^{a}	27.9±1.2	6.5±0.3^{a}	97.0±4.4	21.9±0.8^{a}	34.2±1.7	7.8±0.2^{a}	36.5±1.7	8.2±0.5^{a}
12	50	8	4.3±0.2^{a}	29.9±1.2	6.8±0.1^{a}	102.7±5.6	23.9±1.0^{ab}	35.6±3.1	7.9±0.4^{a}	37.9±2.8	8.3±0.4^{a}
12	100	6	4.4±0.1^{a}	31.4±1.2	7.2±0.3^{a}	100.6±4.0	23.0±0.8^{ab}	36.7±1.1	8.4±0.3^{ab}	38.2±2.8	8.7±0.7^{a}
12	150	8	4.2±0.1^{a}	29.3±1.2	7.0±0.3^{a}	107.1±5.5	25.5±0.9^{b}	37.2±1.8	9.0±0.3^{b}	37.1±2.9	8.7±0.4^{a}

Figure 1. Differences in heart mass occurring in the Incubation Day 0 and 9 treatment groups after 19 days of incubation. For both graphs, the white column represents the sham treatment (0.9% saline) and the black columns represent imidacloprid treatments (solid dissolved in 0.9% saline vehicle) ranging from 10 to 150 mg/kg. Graph A shows the average ratio of heart mass to embryonic mass at ID 0, and B shows the average ratio of heart mass to embryonic mass at ID 9. Dissimilar letters indicate statistically distinct values where noted ($P<0.05$). Data presented as mean ± SE. Sample sizes are included in Table 1.



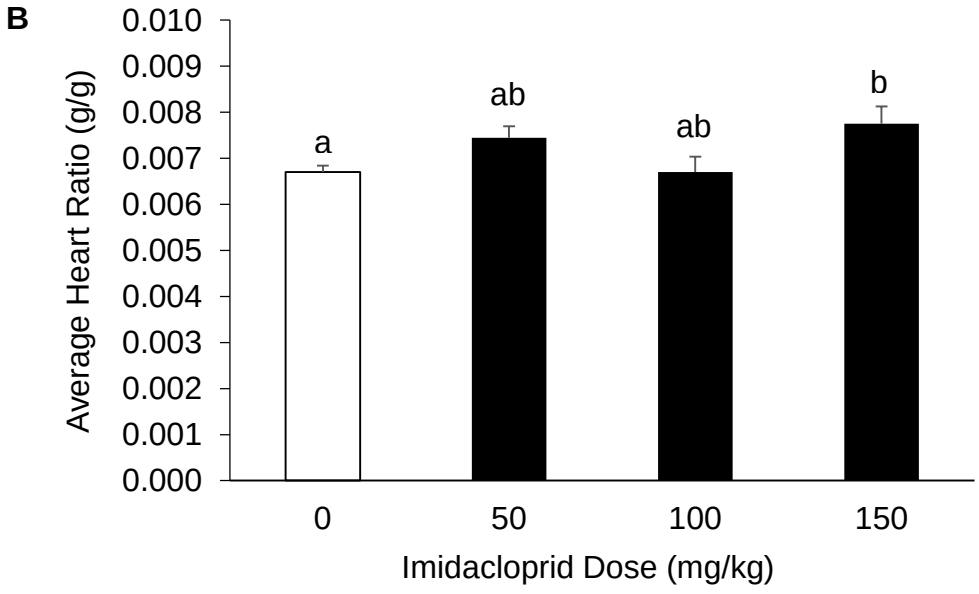


Figure 2. Differences in lung mass occurring in the Incubation Day 0 and 12 treatment groups after 19 days of incubation. For both graphs, the white column represents the sham treatment (0.9% saline) and the black columns represent imidacloprid treatments (solid dissolved in 0.9% saline vehicle) ranging from 10 to 150 mg/kg. Graph A shows the average ratio of lung mass to embryonic mass at ID 0, and B shows the average ratio of lung mass to embryonic mass at ID 12. Dissimilar letters indicate statistically distinct values where noted ($P<0.05$). Data presented as mean ± SE. Sample sizes are included in Table 1.

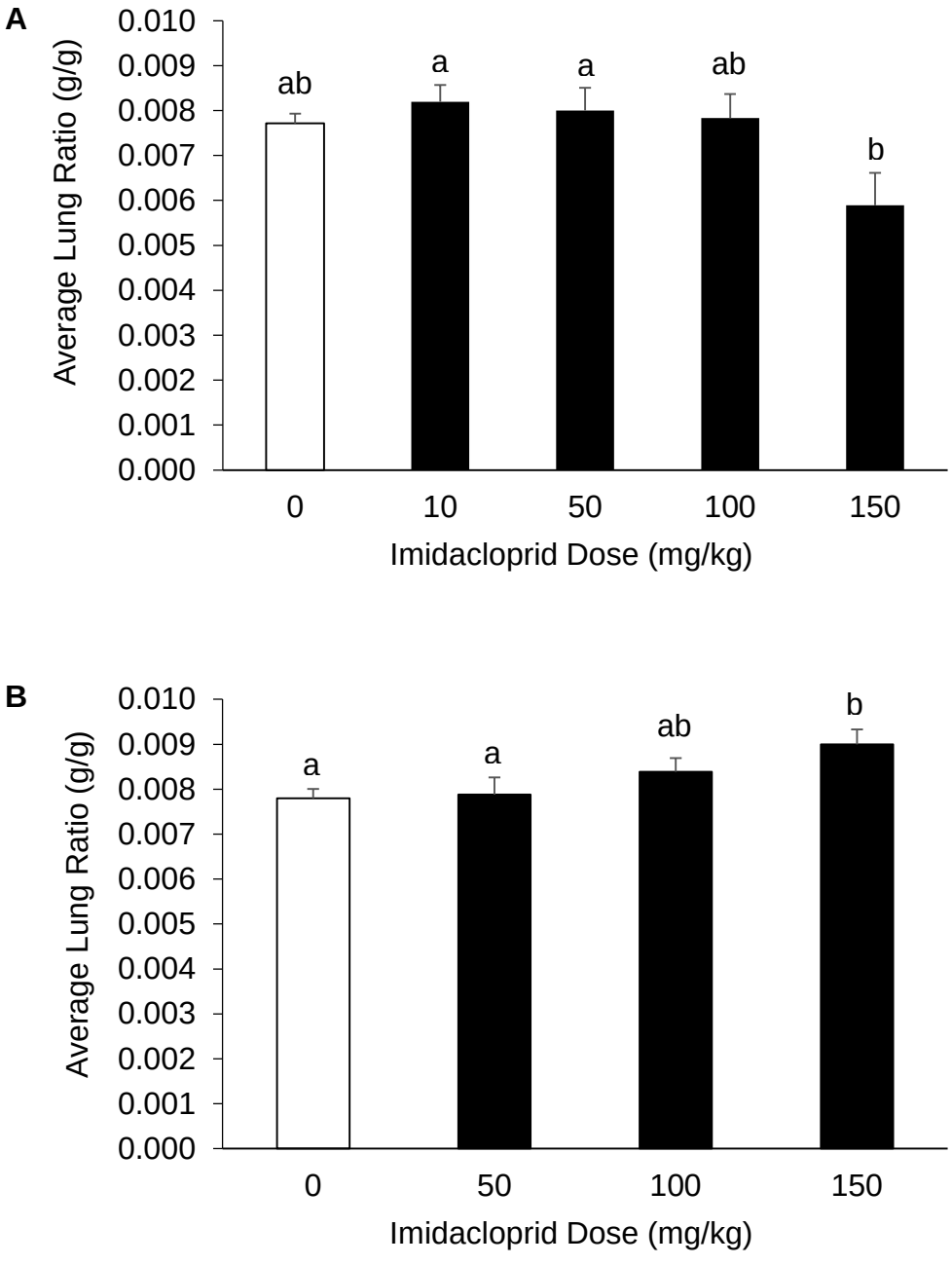


Figure 3. (Below) Differences in liver mass occurring in the Incubation Day 6 and 12 treatment groups after 19 days of incubation. For both graphs, the white column represents the sham treatment (0.9% saline) and the black columns represent imidacloprid treatments (solid dissolved in 0.9% saline vehicle) ranging from 10 to 150 mg/kg. Graph A shows the average ratio of liver mass to embryonic mass at ID 6, while B shows the average ratio of liver mass to embryonic mass at ID 12. Data presented as mean ± SE, and sample sizes are included in Table 1.

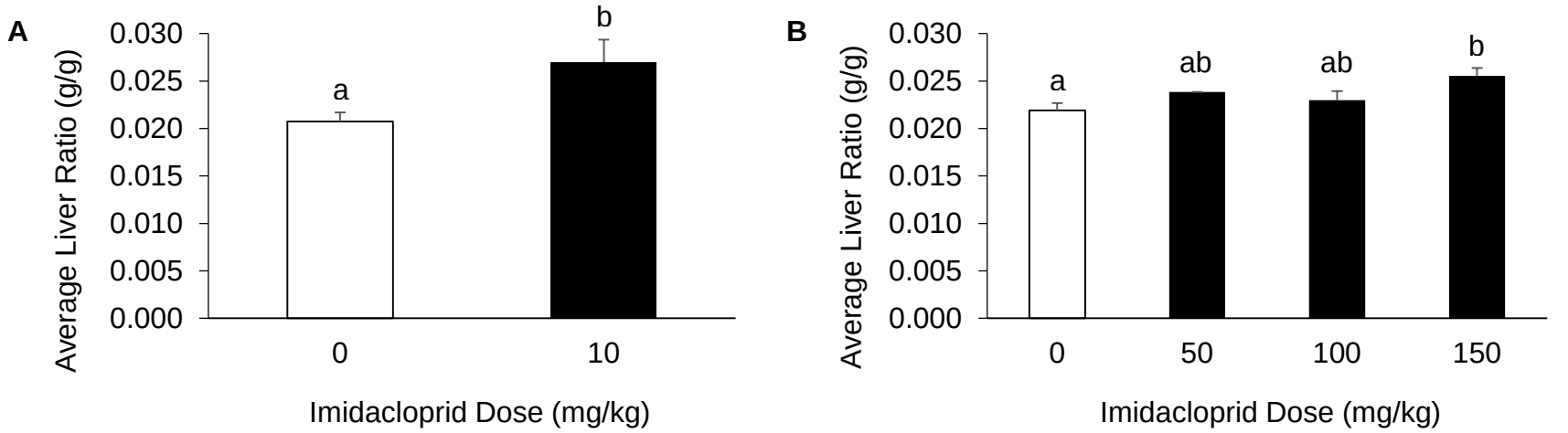


Figure 4. (Right) Differences in kidney mass occurring in the Incubation Day 6 treatment groups after 19 days of incubation. The white column represents the sham treatment (0.9% saline) and the black column represents imidacloprid treatment (solid dissolved in 0.9% saline vehicle) at a concentration of 10 mg/kg. Average ratio of kidney mass to embryonic mass (g/g) is shown. Dissimilar letters indicate statistically distinct values where noted (P<0.05). Data presented as mean ± SE. Sample sizes are included in Table 1.

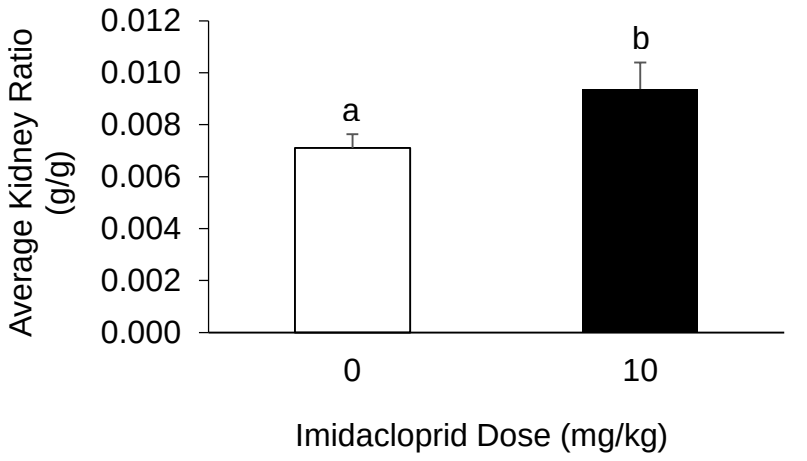
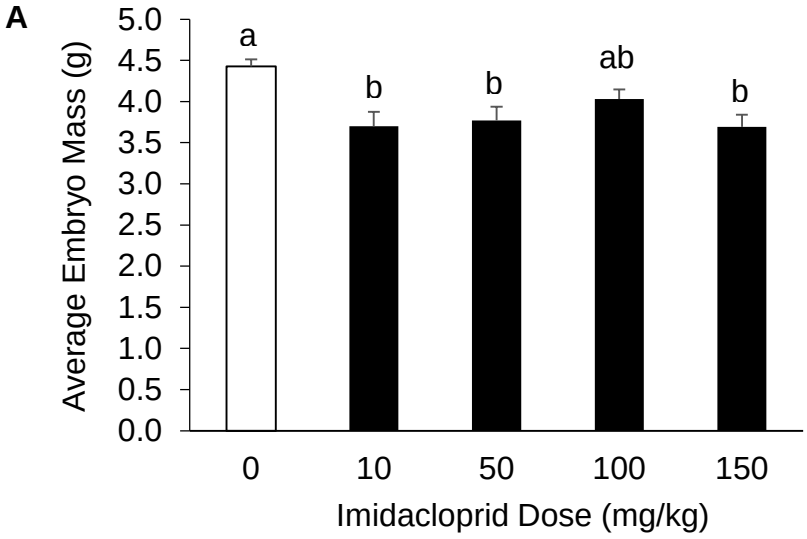
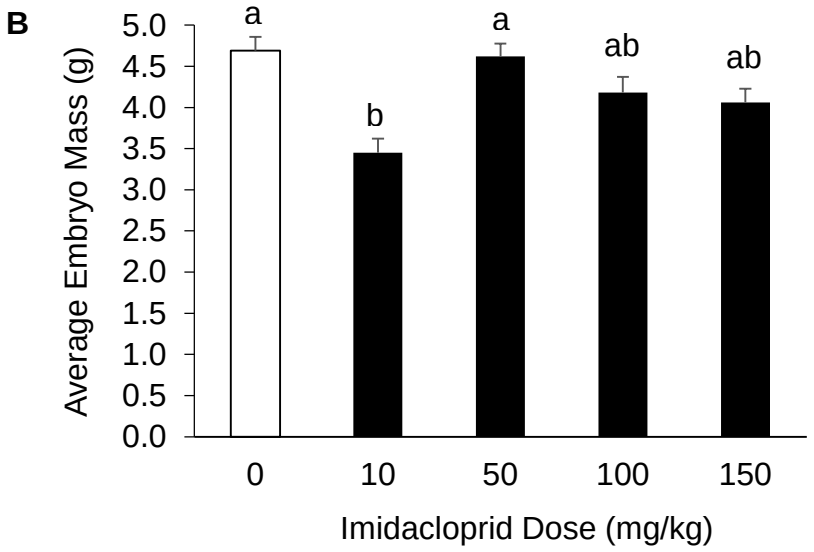


Figure 5. Differences in embryonic mass occurring in the Incubation Day 0, 3 and 9 treatment groups after 19 days of incubation. For all graphs, the white column represents the sham treatment (0.9% saline) and the black columns represent imidacloprid treatments (solid dissolved in 0.9% saline vehicle) ranging from 50 to 150 mg/kg. Graph A shows the average embryonic mass at ID 0, B shows average embryonic mass at ID 3, and C shows average embryonic mass at ID 9. Dissimilar letters indicate statistically distinct values where noted ($P<0.05$). Data presented as mean ± SE. Sample sizes are included in Table 1.





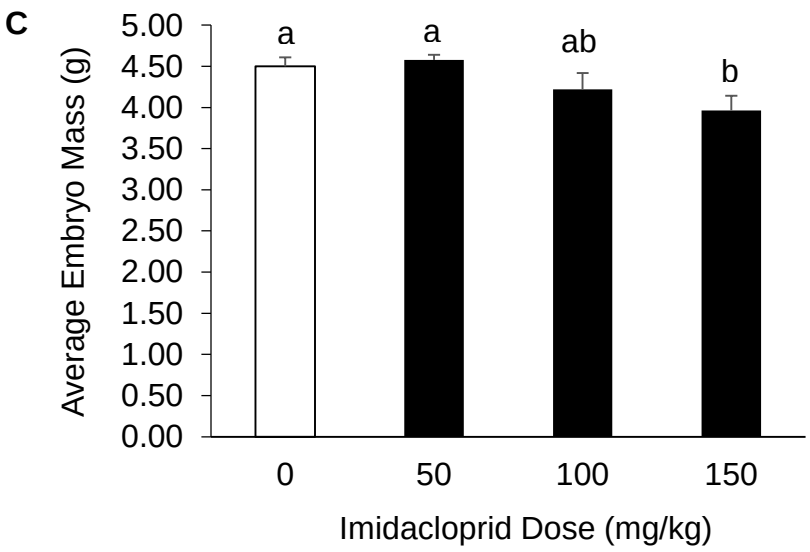
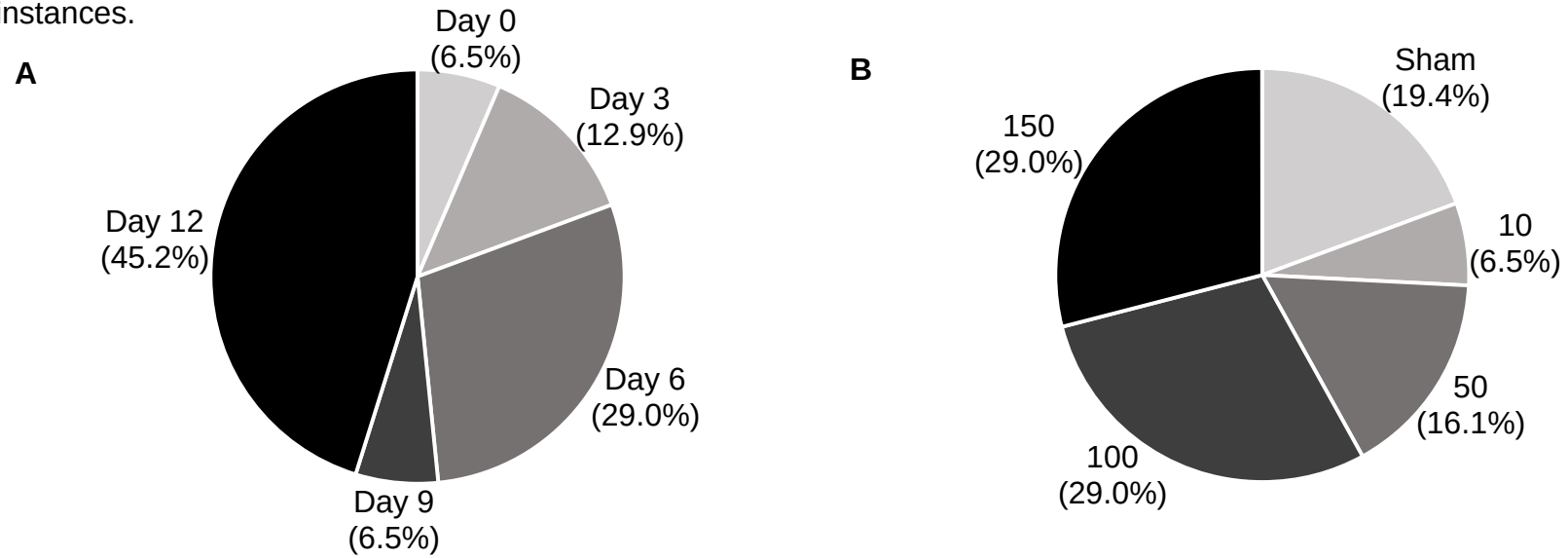


Figure 6. Examples of the deformity types from left to right: abnormal beak, legs, eyes, and an underdeveloped embryo.

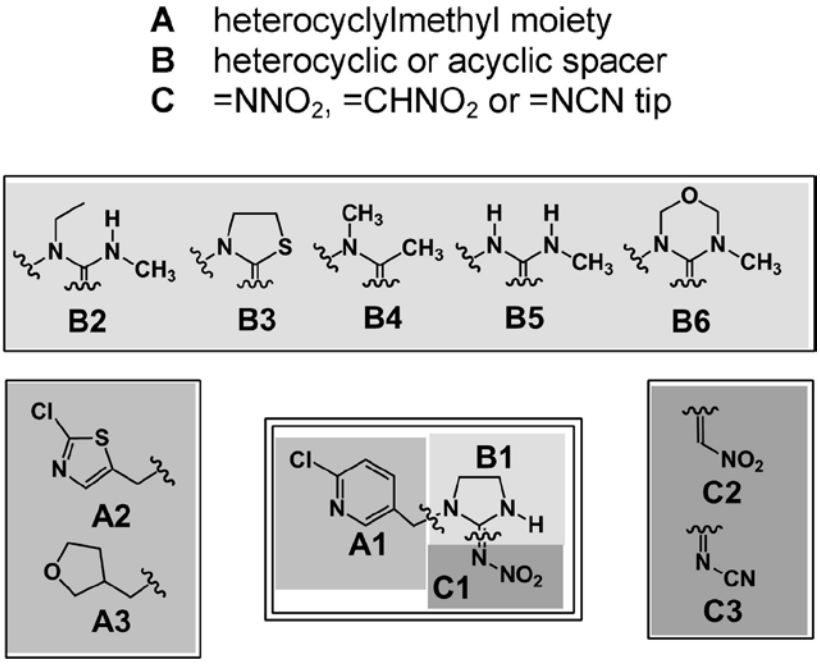
Figure 7. Percent of total deformities occurring at each Incubation Day (Chart A) and Dosing Concentration (Chart B) in experiment 2. Deformity types included malformations of the beak, legs, eyes, and severe underdevelopment for a total of 31 cases. Percentages are equal to the number of cases (of any type) occurring in that treatment out of the 31 total instances.



APPENDIX

NEONICOTINOID MOLECULAR STRUCTURE

General molecular structure for neonicotinoid compounds, showing the three main components and possible variations, with imidacloprid pictured in the middle. Diagram acquired from (Casida, 2011).



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