EXAMINING THE RELATIONSHIP OF PESTICIDES AND AMPHIBIANS IN COSTA RICAN ARTIFICIAL WETLANDS: FROM INDIVIDUALS TO COMMUNITIES

by

KRISTY MARIE SEGAL MCDOWELL

(Under the Direction of C. Ron Carroll and Sonia M. Hernandez)

ABSTRACT

Destruction of natural wetlands is among the largest threats to amphibians. While artificial wetlands have been proposed as a surrogate habitat for displaced amphibians, this has never been rigorously tested in the field. Artificial wetlands expose amphibians to many potential stressors, including pesticides. We assessed variables at the community, population, and individual levels for amphibians in artificial wetlands along a gradient of pesticide exposure in Guanacaste, Costa Rica. Our objective was to determine if pesticide exposure predicted measureable differences in health, population density, and community composition. We did this by assessing the general amphibian community and by a more in-depth study of one species, *Rhinella marina*. The community study detected thirteen species through call sampling and capture. Community composition was similar among sites, but sites with high pesticide application rates had lower community similarity through time. Higher population densities were associated with lower pesticide application rates for two species. Variable species responses are likely due to differences in life history. Pesticides significantly predicted body condition for one of the six species assessed, *Leptodactylus fragilis*, with higher body condition associated with lower pesticide application rates. For *Rhinella marina*, pesticide application rate significantly

predicted the presence of insect head capsules in toad gut contents. Pesticide application rate also significantly predicted infection intensity for several parasites: *Rhabdias* nematodes, intestinal trematodes, haemogregarines, and microfilaria. Higher haemogregarine burdens were associated with higher pesticide application rates, while burdens for the other three parasites showed the opposite trend. All individuals tested were negative for Bd and ranavirus. Taken together, this study suggests that pesticide exposure may be important for amphibians, but importance varies among species. Contrary to expectations, *Rhinella marina* from high pesticide sites were not immunosuppressed. Lower infection intensities of several important parasite species may result in overall better health in sites with higher pesticide application rates. However, lower population densities and species loss from the communities in higher pesticide sites indicate that pesticides could negatively affecting amphibians in other ways. More research is needed to determine the cause of these community level changes, and to make management recommendations to improve artificial wetlands as amphibian habitat.

INDEX WORDS: Cane toad, Amphibian, Pesticide, Costa Rica, Ecoimmunology, Ecotoxicology

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Pesticide usage is a pervasive anthropogenic impact on ecosystems, and will likely increase as agricultural land use becomes more widespread (Foley et al. 2007; Ramankutty et al. 2008). Among other negative side-effects, pesticides can have harmful effects on non-target organisms (Rhind 2009; Rohr et al. 2006). One non-target group of particular concern is amphibians, a group that is already rapidly declining worldwide for a variety of reasons, including habitat loss, disease, and pesticide exposure (Alford and Richards 1999; Stuart et al. 2004).

Pesticides have been associated with a myriad of effects in amphibians and other organisms, from increased pathogen and parasite susceptibility (Forson and Storfer 2006; Kelehear et al. 2009), to indirect effects through trophic cascades (Relyea and Diecks 2008), to reproductive problems (Hayes et al. 2003; McCoy et al. 2008), and death (Davidson and Knapp 2007; Mann et al. 2009). For all of these reasons, pesticides are considered as a stressor for amphibians. What follows is an introduction to the dissertation, and a review of key references on the effects of pesticides on amphibians. The review does not include information that is specific to individual chapters, such as the life history of specific amphibians.

Mortality

To study mortality, the usual procedure is to study the effects of a range of environmentally relevant concentrations for 96 hours and report the LC50 for that time frame; that is, the lethal concentration for 50 percent of the population. These studies are useful as a

starting point but do not adequately represent natural exposure. For example, an often-cited study in Australia (Mann and Bidwell 1999) exposed four species of southwestern Australian frogs to five different formulations of glyphosate. These tests lasted only 48 hours and resulted in low LC50 values. Relyea and Jones (2009) determined the 96-hour LC50 for 13 species of larval amphibians when exposed to Roundup Original Max. They found that lethal concentration varied by species, and that salamanders were generally less sensitive than anurans. In natural ecosystems, amphibians might be exposed for longer periods of time than the duration of these studies, and most likely would experience pulsed repeated exposure (Thompson et al. 2004). This inconsistency is a major flaw in the design of laboratory studies designed to assess mortality.

Other studies have taken a more realistic approach to pesticide exposure. Relyea (2005) performed mesocosm experiments with three species of tadpoles and a direct overspray of Roundup at environmentally relevant concentrations, and laboratory experiments with three species of juvenile amphibians and the same over-application of Roundup. In both experiments high levels of mortality were observed (64-100%). Davidson and Knapp (2007) studied the effects of both pesticide exposure and introduced predatory fish on mountain yellow-legged frogs (*Rana muscosa*). They found that pesticides and fish were both significant predictors of the distribution of the frogs, and that shelter from the wind was an important landscape variable for the frogs, as a protection from wind-borne pesticides.

Growth and Metamorphosis

A variety of studies have examined the effects of pesticides on larval amphibians. Often, time to metamorphosis is extended or other alterations in growth occur as a result of pesticide exposure (Baker et al. 2013; Cheek et al. 1999). Relyea (2004) examined the effects of four

pesticides on the growth and survival of five amphibian species. Effects were dose dependent, but growth was reduced in 35-70% of treatments. Hayes et al. (2006) performed a laboratory study with nine pesticides using leopard frogs (*Rana pipiens*). Both growth and development were impacted, and exposed larvae took longer to metamorphose but were still smaller at metamorphosis. A study on Roundup with four amphibian species found that effects varied by species and developmental stage. *R. pipiens* had the most substantial effects, with decreased snout-vent length at metamorphosis and increased time to metamorphosis (Howe et al. 2004). Ortiz-Santaliestra and Sparling (2007) found that perchlorate inhibited metamorphosis at low doses and increased mortality at high doses. While effects differ among pesticides and species, changes in growth and development are some of the most commonly reported effects of pesticide exposure on amphibians.

Immune Function

Laboratory studies have demonstrated that pesticides can alter multiple aspects of amphibian immune function (Christin et al. 2003; Christin et al. 2004; Gilbertson et al. 2003; Hayes et al. 2006), and alter an individual's susceptibility to a specific parasite (Forson and Storfer 2006; Kerby and Storfer 2009; Rohr et al. 2008). Pesticides can downregulate immune function through the stress response, causing neuroendocrine changes that result in immunosuppression, or through acting directly on the immune system itself (Carey et al. 1999). Gilbertson et al. (2003) tested the response of the humoral, innate, and cell-mediated immune responses of *R. pipiens* to injections of DDT, malathion, and dieldrin. They discovered that immune activity changed across the board in response to pesticide injections. Complementary field studies found differences in immune function between pesticide exposed and pesticide free locations. If the negative effects on the immune system are large enough, pesticides can lead to

increased parasite burdens in exposed amphibians. Linzey et al. (2003) performed field collections of amphibians in Bermuda and tested for immune function and trematode infections. Soil and water samples were collected from the same locations to assay pesticide and heavy metal concentrations. They found that immunosuppression and increased trematode infection prevalence were both associated with contaminated locations.

Reproduction

Many studies have found that pesticide exposure can alter amphibian reproduction function. Atrazine has been associated with increases in hermaphroditism (Hayes et al. 2003) and can affect sexual differentiation (Tavera-Mendoza et al. 2002; Tavera-Mendoza et al. 2002). McCoy et al. (2008) found both increased feminization and increased intersex characteristics in cane toads (*Rhinella marina*) in sites with a high percentage of agricultural land use. It is possible that these effects could lead to differential reproductive success in habitats with high pesticide exposure, which could affect the long-term survival of these populations.

Indirect Effects

Pesticides can also affect amphibians through indirect effects on the wider community. Relyea et al. (2005) examined the indirect effects of Roundup and malathion in a community of tadpoles, zooplankton, algae, predatory newts and larval beetles. While the direct mortality effects on the tadpoles were large, as expected, the researchers found no indirect effects with Roundup due to differential predator survival or algal abundance. However, malathion resulted in an increase in tadpole survival and biomass due to mortality of the predatory larval diving beetles. Ingermann et al. (2002) found that methoxychlor affected both larval salamanders and dragonfly naiads, but that larval salamanders were affected at lower concentrations, resulting in increased predation on larval salamanders by naiads.

Effects of pesticides can vary among community members, and among pesticides. A mesocosm study performed by Rohr and Crumrine (2005) examined the effects of atrazine and endosulfan on a pond community. Effects on the biota were complex, and the pesticides resulted in both direct and indirect effects on various community members. The effects were altered due to the presence or absence of wood frog tadpoles (*Rana sylvatica*), caged dragonfly larvae, and adult snails. Endosulfan had a net positive effect on tadpoles, while atrazine had a net negative effect. Similarly, Relyea and Diecks (2008) used a mesocosm study containing zooplankton, phytoplankton, periphyton, and larval *R. sylvatica* and *R. pipiens* to examine the effects of malathion. Zooplankton were killed by the insecticide, resulting in a trophic cascade. Larval leopard frogs suffered high mortality due to slow growth and development; however, larval wood frogs were largely unaffected due to faster development time. A study by Vonesh and Kraus (2009) allowed natural colonization of experimental pools with and without carbaryl and overall, species richness was higher in contaminated pools. The authors suggest that the presence of pesticides may change the community composition on which their effects play out, which could have significant consequences given the importance of community context to evaluating the net effects of pesticides in natural ecosystems.

Interactions with Other Stressors

Pesticides can also interact with a variety of environmental factors, including parasites (Forson and Storfer 2006; Kerby and Storfer 2009; Kerby et al. 2011) to create different effects on the organism of interest. One way pesticides and parasites may interact is through downregulation of a host's immune system (Christin et al. 2003; Christin et al. 2004; Gilbertson et al. 2003; Lafferty and Kuris 1999; Rohr et al. 2008). Both increased macro-parasite burdens and altered effects of parasite infection have been shown in experimental exposures of

amphibians to various pesticides (Kiesecker 2002; Rohr et al. 2008; Rohr et al. 2008), as well as some field studies (Linzey et al. 2003). Pesticides also increase susceptibility to, and subsequent mortality from, at least one amphibian micro-parasite, *Ambystoma tigrinum* virus, a ranaviral pathogen (Forson and Storfer 2006; Kerby and Storfer 2009). Limb deformities caused by trematode infections may also be affected by pesticide exposure. Kiesecker (2002) found that while trematode infection was necessary to cause limb deformities, deformities were more common at sites near agricultural runoff.

Pesticides can also interact with other forces, such as predatory stress (Kerby et al. 2011; Relyea 2005; Relyea 2006), and other contaminants (Kerby and Storfer 2009; Ortiz-Santaliestra and Sparling 2007). Kerby et al. (2011) discovered that larval salamander survival was lowest when they were exposed to carbaryl, dragonfly predator cue, and the *Ambystoma tigrinum* virus. Ortiz-Santaliestra and Sparling (2007) studied the effects of nitrate and perchlorate on southern leopard frogs (*Rana sphenocephala*). They found that while both contaminants affected development and survival, together they had additive effects. Relyea (2009) used mesocosm experiments to test the effects of ten different pesticides on gray tree frogs (*Hyla versicolor*) and *R, pipiens*, both singly and in mixtures. He found that the pesticides resulted in a wide range of both direct and indirect effects, and that the effects of mixtures could not be predicted by effects of single pesticides.

Problem Statement

As agricultural land use continues to increase (Foley et al. 2007; Ramankutty et al. 2008), pesticide usage is likely to also increase. At the same time, wetlands are the fastest declining habitat on the planet (Millenium Ecosystem Assessment 2005; Prigent et al. 2012). This combination of factors forces many amphibians to utilize habitats contaminated with pesticides

and other anthropogenic chemicals as an alternative for natural wetlands. It is not well understood if these habitats perform as surrogates for natural wetlands for some species, and what the consequences of chemical exposure can be over long periods of time in field environments. Because amphibians are the fastest declining vertebrate group (Stuart et al. 2004), it is crucial that we understand their probability of long-term success in human-modified environments if we hope to conserve them.

Research Objectives

This study examines assemblages of amphibians living in artificial wetlands in Costa Rica under a suite of pesticide application regimes to determine the relationship of these chemicals with amphibian health, population density, and community composition. We assessed both the general amphibian community and conducted an in-depth study of one species, *Rhinella marina*.

Study Area

The Rio Tempisque watershed in Guanacaste, Costa Rica is approximately 5,404 sq km extending from the central mountains to the Pacific coast. Land use in the watershed is a mosaic of urban areas, protected areas, and various agricultural crops, with rice being one of the most widespread.

Sample sites consisted of artificial wetlands in the Rio Tempisque watershed near the town of Cañas (Appendix A). We defined artificial wetlands as any area that is flooded due to human activities, whether permanently, such as a pond, or temporarily, such as in agriculture. Artificial wetlands used as sample sites included four rice fields, a seasonally flooded woodlot, and an artificial pond and surrounding lawn on a hotel grounds (Appendix B). All sampling took place during the transition from the dry season to the wet season (March to June) in 2011 – 2012,

because the beginning of the wet season is the peak breeding period for most local amphibian species.

Sample sites varied in their amount of pesticide exposure. Pesticide exposure was estimated using landowner surveys, as explained below. Pesticide exposure varied from none reported to levels typical of commercial rice production. This range of pesticide exposure provided a gradient of exposures in habitats that in all other regards are quite similar.

Methodology

Landowner/Manager Surveys

We contacted the landowners or managers of all sample sites personally. Each landowner or manager answered standardized survey questions regarding the types and quantities of pesticides applied (Appendix C). These data (Appendix D) were then used to calculate the total pesticide application rate per hectare per year for each separately managed site.

Soil Pesticide Concentration Analysis

Soil samples were collected in May - July of 2012. Two samples were collected from each field site, approximately five weeks apart. Samples were a composite collected from the top 25 mm of soil at five locations around the field sites, from areas that were moist but not waterlogged. The soils were placed in 75 mL plastic containers and stored at -20 ^{o} C until export to the UGA Agricultural Services Laboratory, Athens, GA. Samples were received at the UGA Agricultural Services Laboratory, assigned individual identification number and stored at – 4^{o} C until analysis. Soil sample composites were soxhlet extracted with ethyl acetate for 4 hrs. The extract was concentrated on rotary evaporator and made to a final volume of 2 ml. for GLC analysis. The extract was then analyzed for nitrogen and phosphorus containing pesticides using a Perkin Elmer Autosystem Gas Chromatograph equipped with a NP detector and a ZB-5

Megapore (0.53mm) 30-m column. The column was programmed from 135 to 275^{o} C at 5^{o} C min^{-1}. A fortified sample and reagent blank was included with the sample extraction set. Potential positive residues were quantified by external standardization (3 point calibration) with the lower reporting limit set equal to the lowest calibration standard. The chromatography is a modification of EPA Drinking Water Method 507 adapted to current laboratory analytical systems.

The extract was also analyzed for chlorinated pesticides using a Agilent 7890 series Gas Chromatograph equipped with dual Ni63 electron Capture detectors and a ZB-5 Megapore (0.53mm) 30-m columns. The column was programmed from 135 to 275^{o} C at 5^{o} C min^{-1}. A fortified sample and reagent blank was included with the sample extraction set. Potential positive residues were again quantified by external standardization (3 point calibration) with the lower reporting limit set equal to the lowest calibration standard. The chromatography is a modification of EPA Drinking Water Method 508 adapted to current laboratory analytical systems.

Amphibian Captures

All procedures were approved by the University of Georgia's Institutional Animal Care and Use Committee (AUP #A2012 03-011). Amphibians were caught opportunistically during 26 sampling dates for the entire community and 62 sampling dates for *R. marina* evenly distributed throughout the sampling period. Fields were walked and an attempt was made to capture any amphibian that was visualized or heard calling.

Community Call Sampling

Call sampling was conducted to assess community composition. It was performed at sunset for a 30-minute period during a total of 47 sampling dates. The same researcher conducted

all call sampling, to minimize sampling bias. Using previously recorded calls of the approximately 15 species in the region as a reference (provided by S. Connelly), all breeding calls heard during the sampling period were identified to species. The presence and absence data from call sampling was supplemented by visual identifications made during captures.

Amphibian Community Analyses

Once caught, individuals were placed in clean plastic bags, identified to species, weighed, and measured. Body condition scores were calculated as weight/snout-to-vent length (SVL) for the six most common species where sufficient sample sizes could be obtained. Because of morphological differences between species, body condition can only be compared within a single species, so each species was analyzed separately. We calculated catch/unit effort for all species, as an index of population densities in each study site. In addition to body condition and catch/unit effort, we also assessed the Bd infection status of a subset of captured amphibians. The ventral surface of each amphibian was swabbed 20 times using a sterile cotton swab to collect DNA for Bd testing following standard sampling techniques (Hyatt et al. 2007). Swabs were frozen -20˚C dry in sterile microcentrifuge tubes until extraction. DNA was extracted from swabs using Qiagen DNeasy kits (Qiagen, Venlo, Limburg, Netherlands) following the manufacturer's directions modified for extractions for swabs, using the methods of M. Yabsley, and the DNA product was tested for Bd by conventional PCR following the methods of (Annis et al. 2004).

Amphibian Community Statistical Analyses

Data on total pesticide application rate were used in analysis rather than quantitative soil pesticide concentrations, because concentrations in soil samples were below detection limits. Total pesticide application rates were calculated as total liters of herbicides and insecticides

applied per hectare per year. The log of total pesticide application rates was used, due to a right skewed distribution. Body condition was analyzed using general linear mixed-effects models with site as a random effect. Fixed effects included total pesticide application rate, year captured, and day of year captured. A stepwise regression was used to eliminate the variable with the highest p-value until the remaining model included only significant effects (p-value$\leq$0.05). Catch/unit effort was analyzed using a general linear mixed-effects model with total pesticide application rate, year captured, day of year captured, species, and species-pesticide interactions as fixed effects, and site as a random effect. A stepwise regression was used to eliminate nonsignificant variables.

Call sampling was analyzed with Nonmetric Multidimensional Scaling (NMDS). For community analysis, NMDS requires Bray-Curtis dissimilarity values, or some other measurement of distance between communities, for every pairwise comparison of observations (Gardiner et al. 2009) so a Bray-Curtis dissimilarity matrix was created using the number of species detected and species identities on each call sampling night. This matrix was then used to plot the communities in an n-dimensional space, while preserving the distances, or Bray-Curtis dissimilarity values, between points. Forty iterations were conducted to ensure an optimal solution. We began with a two dimensional analysis, and added dimensions until there was not a meaningful change in the plot. We assessed the within site variance in community composition more closely using the within site Bray-Curtis dissimilarities. These values were analyzed using a one-way ANOVA and a Tukey's Honestly Significant Difference (HSD) test. Bd infection status was not analyzed, as no amphibians were found to be positive for Bd. All analyses were completed using the program R version 3.0.1 with package lme4.

Rhinella marina *Necropsies*

R. marina were held in individual enclosures for 12-36 hours before necropsy, during which time pre-necropsy samples were taken. Toads were humanely euthanized following approved methodology. Briefly, toads were immersed in solution containing an overdose of buffered tricaine methanesulfonate (MS-222), followed by cervical pithing (American Veterinary Medical Association 2007).

Rhinella marina *Corticosterone Analysis*

Blood for corticosterone analysis was collected immediately upon capture from the abdominal midline vein for a baseline CORT measurement. All blood samples were collected within three minutes of capture; if this was not possible, no blood sample was analyzed for that individual to avoid measuring an increase in corticosterone levels associated with capture stress (Busch and Hayward 2009; Romero and Wikelski 2001). Corticosterone was extracted from plasma samples with an ether extraction. Briefly, 1800 cpm of tritiated corticosterone was added to each sample for later recovery calculation. Next, 3 mL of diethyl ether was added to each sample, the mixture was vortexed for 30 seconds and allowed to settle for 20 minutes. Samples were then snap frozen and the supernatant was poured off and dried using an N_2 stream. Corticosterone was quantified using standard competitive binding radioimmunoassays (using Anti-CORT from MP Biomedicals, Solon, OH) as described in Wingfield and Farner (1975). All samples were done in one assay for each hormone. Average recovery was 85% and intrassay variation was 4.71% for 2011 samples, and 70% average recovery and 2.16% intrassay variation for 2012 samples.

Rhinella marina *Righting Reflex Test*

The righting reflex test was conducted following an 18-hour acclimation period after capture. The toad was flipped onto its dorsal side on a hard counter, and the timed trial began. The toad was allowed to right itself and then was immediately flipped over again. This continued until the toad could no longer right itself. If the toad could not right itself within one minute after being flipped, the trial was ended. Both the total time of the test and the number of times the toad righted itself were recorded, and each variable was analyzed separately.

Rhinella marina *PHA Test*

After capture, the thickness of the second toe web on the right hind foot was measured with digital calipers. The toe web was then injected with 50 uL of 2mg/mL PHA dissolved in phosphate buffered saline (PBS). Injections were made with 27 gauge tuberculin syringes to minimize inflammation due to injection. Toe web thickness was measured at 6, 12, and 24 hours post injection to monitor inflammatory response. All measurements were made three times and averaged for improved accuracy, and the average pre-injection toe web thickness was subtracted from this value to provide an average swelling response for each time point. Only the 12-hour time point was analyzed, because this was the height of the swelling response. To determine the inflammatory effects of PHA at the cellular level, a four mm sample of the toe web where PHA was injected was collected during necropsy and preserved in 10% buffered formalin. Toe webs were examined and scored for inflammation by a board-certified veterinary pathologist with expertise in herpetofauna. The following scoring system was used: 0 = no significant lesion observed; 1 = edema and/or hemorrhage; 2 = cellular infiltrates.

Rhinella marina *Body Condition Measurements*

Body condition measurements were made using several different methods. Each individual was weighed and snout-vent length (SVL) measured. Body condition scores were calculated as weight/SVL. Parotid glands were also measured, due to the physiological cost associated with producing the toxin in the glands. To calculate the area of the parotid gland, the longest axis of the gland and the widest perpendicular axis were measured. This area was then adjusted for the size of the toad by dividing by SVL. One gland was measured for each toad. The final metric we used for body condition was the fat body weight. Fat bodies are a more direct way to measure the condition of an amphibian, since a body condition score could be skewed by the reproductive status of a female or the time since the toad's last meal. *R. marina* have discrete fat bodies within their body cavity and during necropsy, all fat bodies were collected and weighed.

Rhinella marina *Gut Content Analysis*

Fecal samples were collected from the terminal section of the gastrointestinal tract during necropsy and preserved in 2.5% potassium dichromate. Samples were sorted for insect head capsules under a dissecting microscope. Only head capsules were counted to provide a conservative estimate of number of insects in the gut contents. Insects were identified to order. Gut contents were analyzed as presence/absence of any head capsules, to avoid inaccurate counts due to deterioration.

Rhinella marina *Parasite Collection and Analysis*

Ectoparasites (ticks) were removed from the skin and preserved in 100% ethanol. Blood was collected from either the abdominal midline vein or the heart, and blood smears were made in a standard manner, air-dried, fixed with 100% methanol, and stained using a modified

Romanowsky staining technique (Diff Quick®, Jorgensen Laboratories, 1450 Van Buren Ave., Loveland, CO 80538, USA). The entire slide was scanned for microfilaria at 100x, and the monocellular layer was examined at 400x and 1,000x (with oil immersion) for 10 minutes for haemoparasites, which were identified to genus based on morpohology (Desser 2001).

To determine the abundance of *Rhabdias* nematodes, the lungs were removed from each toad and fixed in 10% buffered formalin until later examination. *Rhabdias* nematodes were then individually removed from the lungs by careful dissection with hemostats and counted. To determine the abundance of intestinal parasites, intestines were linearized, opened lengthwise, scraped and washed with 100% ethanol into a 100 mesh. The mesh contents were then preserved in 100% ethanol. Intestinal wash samples were subsequently examined under a dissecting microscope, and all intestinal parasites were counted and identified to phylum.

Rhinella marina *Bd and Ranavirus Sample Collection and PCR*

The ventral surface of each toad was thoroughly swabbed 20 times using a sterile cotton swab to collect DNA for Bd testing following standard sampling techniques (Hyatt et al. 2007). Swabs were frozen at -20˚C dry in sterile microcentrifuge tubes. A 25 mg section of the liver was collected during necropsy and frozen at -20˚C for ranavirus testing. DNA was extracted from both swabs and liver tissue using Qiagen DNeasy kits (Qiagen, Venlo, Limburg, Netherlands) following the manufacturer's directions, and DNA products were tested for Bd or ranavirus by conventional PCR using established methods (Annis et al. 2004; Greer and Collins 2007; Mao et al. 1997).

Rhinella marina *Histopathology*

To look for cellular level effects of parasite infection, tissue sections of all the major organs were collected during necropsy and preserved in 10% buffered formalin. Histopathology

slides were examined and scored by a board-certified veterinary pathologist with expertise in herpetofauna. The total number of organs showing effects of parasites and the number of different parasite taxa identified were counted for each toad. Effects of trematodes in the liver were scored by the following system: 0 = no significant lesion observed; 1 = trematodes and/or bile duct dilation only (no inflammation, hyperplasia, or fibrosis); 2 = biliary epithelial hyperplasia, branching, or inflammation; 3 = peribiliary fibrosis.

Rhinella marina *Statistical Analysis*

Total pesticide application rate was used to analyze data rather than quantitative soil pesticide concentrations, because concentrations in soil samples were below detection limits. Total pesticide application rates were calculated as total liters of herbicides and insecticides applied per hectare per year. The log of total pesticide application rates was used, due to a right skewed distribution. Most models were general linear mixed-effects models with site as a random effect. Fixed effects varied depending on the response variable, but total pesticide application rate, year captured, day of year captured, and sex of individual were always included. The only exceptions to this were that year captured was not included for haemogregarines or microfilaria, because burdens for these parasites were only assessed for toads captured in 2012, or for either of the righting reflex variables, since this test was only performed on toads captured in 2012, and day of year captured was not included for toe web histopathology scores, due to constraints of the ordinal model. Two sets of models were run for each response variable, one including only the fixed effects mentioned above (referred to as basic model), and one including other effects that could have biological relationships with the response variable (referred to as full model). The number of samples per predictive variable was always ≥10, to avoid overparameterization of the models (Vittinghoff and McCulloch 2007). A Pearson Correlation

matrix was completed to ensure that auto-correlated variables were not included as effects in the same model. For each set of models, a stepwise regression was employed to eliminate the variable with the highest p-value until the remaining model contained only significant effects (p-value≤0.05). However, marginally significant p-values (≤0.1) for total pesticide application rate are reported. Bd and ranavirus infection status were not analyzed, since all individuals tested were negative for both pathogens. Generally, linear regressions were used, but a logistic regression was used for gut contents analysis, and an ordinal logistic regression was used for toe web histopathology analysis and liver lesions histopathology analysis. All analyses were completed using the program R version 2.14.1 with package lme4, except the toe web and liver lesions histopathology analyses, which were completed with package ordinal.

Summary of Enclosed Manuscripts

Chapter 2 "Relationship of pesticides and community composition, population density, and individual body condition and Bd infection status of amphibians in rice fields" is a study that spans several levels of organization to provide a comprehensive assessment of the state of the amphibian community in rice fields along a gradient of pesticide usage in Guanacaste, Costa Rica.

Chapter 3 "Relationship of pesticides and *Rhinella marina* immune function and body condition" uses one species of amphibian, the cane toad, to examine the relationship of pesticide exposure with immune function and body condition.

Chapter 4 "Relationship of pesticides and *Rhinella marina* macro- and micro-parasite burdens" uses the same species and investigates the relationship of pesticide exposure with parasite prevalence and infection intensity.

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CHAPTER 2

RELATIONSHIP OF PESTICIDES AND COMMUNITY COMPOSITION, POPULATION DENSITY, AND INDIVIDUAL BODY CONDITION AND BD INFECTION STATUS OF AMPHIBIANS IN RICE FIELDS[1]

[1]McDowell, K. M. S., S. M. Hernandez, and C. R. Carroll. To be submitted to Biotropica.

Abstract

Pesticides are a major stressor for amphibians living in human impacted habitats, and this stress can lead to effects at the individual, population, and community levels. To understand the relationship of different pesticide regimes with natural assemblages of amphibians, we assessed the body condition, *Batrochochytrium dendrobatidis* (Bd) infection status, population density, and community composition of amphibians living in artificial wetlands in Guanacaste, Costa Rica with a variety of pesticide application rates. Our objective was to determine if pesticide exposure was associated with changes in any of these variables. Thirteen species of amphibians were detected by a mixture of call sampling and capture. Community composition was similar among sites, but the similarity of the community through time varied, with sites with high pesticide application rates generally having lower community similarity through time. Population densities, as measured by catch/unit effort, were not affected by total pesticide application rate for the community overall. However, for two species, higher population densities were associated with lower pesticide application rates. These variable responses among species are most likely due to differences in life history characteristics. Of the six species assessed, only the body condition of *Leptodactylus fragilis* was significantly related to pesticide application rate, with higher body condition associated with lower pesticide application rates. All individuals tested were negative for Bd. Taken together, this suggests that pesticide usage may be an important habitat feature for amphibians, but importance varies among species in a community. Further research is needed to determine the mechanism(s) by which pesticides may be affecting amphibian communities, and to determine potential management strategies to mitigate these effects.

Introduction

As agricultural land use becomes more widespread than ever (Foley et al. 2007; Ramankutty et al. 2008), pesticide usage will likely continue to increase. Among others, the negative side-effects of pesticides can include harmful effects on non-target organisms (Rhind 2009; Rohr et al. 2006). One non-target group of particular concern is amphibians, a group that is already rapidly declining worldwide for a variety of reasons including habitat loss, disease, and pesticide exposure (Alford and Richards 1999; Stuart et al. 2004).

Most studies on the impacts of pesticides on amphibians do not examine their effects in situ. They either measure mortality under experimental conditions, (Bruhl et al. 2013; Kerby and Storfer 2009; Mann and Bidwell 1999; Mann and Bidwell 2001), or, more recently, measure both lethal and sublethal effects through mesocosm studies (Cothran et al. 2013; Relyea 2006; Relyea 2009; Relyea and Diecks 2008; Rohr and Crumrine 2005; Rohr et al. 2008). While mesocosm experiments more accurately mimic natural conditions and can assess indirect effects, few studies have taken place in natural assemblages under existing pesticide regimes (but see Hayes et al. 2003; Kiesecker 2002; Linzey et al. 2003; McCoy et al. 2008; McCoy et al. 2008; Rohr et al 2008). Pesticides can interact with other forces, such as predatory stress (Kerby et al. 2011; Relyea 2006), pathogens (Kerby and Storfer 2009; Kerby et al. 2011; Rohr et al. 2008), and other contaminants (Kerby and Storfer 2009; Ortiz-Santaliestra and Sparling 2007; Relyea 2009) to create different effects on the organism of interest. To gain a more complete understanding of the impacts of pesticides, studies in natural systems are a critical addition to laboratory and mesocosm studies.

The impacts of pesticide usage on amphibians are of particular concern in Central America, where some of the highest diversity in the world exists. This region has also

experienced the most devastating amphibian declines, as a result of both land use changes and disease, most notably the fungus *Batrachochytrium dendrobatidis* (Bd). Bd is the causative agent of a disease that is causing massive die-offs of amphibian populations worldwide, and has been particularly lethal to amphibians in Central America (Daszak et al. 1999; Fisher et al. 2009).

Simultaneously, Central American countries import an average of 33 million kg of pesticide active ingredients per year, and this number continues to grow (Bravo et al 2011). Specifically, Costa Rica, a country dependent on ecotourism and dedicated to conservation, imports more pesticides than any other Central American country (Bravo et al 2011), and more pesticides per hectare than any other country in the world (World Resources Institute 2011). Costa Rica is home to 174 species of amphibians, 44 of which are endemic (Savage 2002). Understanding how pesticides impact amphibians in this complex natural system is critical to forecasting how they will persist in the future, and maintaining this incredible biodiversity.

Determining the impacts of pesticide usage is particularly important for understanding the health of amphibians due to the increase of amphibian dependence on artificial wetlands. Wetlands are the most threatened habitat type worldwide (Millenium Ecosystem Assessment 2005), and have declined by 6% in 15 years (Prigent et al. 2012), forcing many amphibians to find alternative habitats. In Costa Rica, particularly in the in the Guanacaste province (Organization for Tropical Studies 2001), rice is a major crop, and will most likely continue to grow in importance in the region. Rice fields are sometimes touted as a useful alternative habitat for many amphibian species where natural wetlands are declining (Duré et al. 2008; Machado and Maltchik 2010). Indeed, in Guanacaste, rice fields are particularly attractive habitat for amphibians, because most of the region is extremely hot and dry for six months of the year, and natural wetlands are scarce and declining due to increased anthropogenic land use (Daniels and

Cumming 2008). In addition, climate change predictions for the region suggest that Guanacaste will become increasingly dry, possibly exacerbating these conditions (Magrin et al. 2007).

Pesticide application in rice fields may be an impediment to amphibian use of these habitats. Pesticides have been shown to cause a myriad of effects in amphibians and other organisms, from increases in susceptibility to pathogens and parasites (Forson and Storfer 2006; Kelehear et al. 2009), to indirect effects through trophic cascades (Relyea and Diecks 2008), reproductive problems (Hayes et al. 2003; McCoy et al. 2008), and mortality (Davidson and Knapp 2007; Mann et al. 2009). While these studies have increased our understanding of the impacts of pesticides on amphibians, few have been performed in the field under natural conditions and in combination with other potential stressors.

We used both call surveys and captures to study natural assemblages of amphibians living in artificial wetlands in Guanacaste, Costa Rica. This approach allowed us to collect data at the individual, population, and community levels, for a more complete assessment of the status of amphibians in these habitats. The advantages of call surveys are that audio sampling is more complete than visual sampling and the researcher is less likely to make identification errors, as breeding calls are often more distinct than visual appearance. However, call sampling cannot provide density estimates, as it is not possible to distinguish individuals, and may underestimate the species present, as all species may not be calling on a given sampling date. For this reason, we also incorporated captures, both as a way to assess relative population density, and to collect individual level data. We assessed body condition and Bd infection status at the individual level. Body condition scores provide a proxy for energy stores, and have been used to estimate the effects of a variety of stressors (Bancila et al. 2010; MacCracken 2005; MacCracken and Stebbings 2012; Reading 2007; Waye and Mason 2008). While Bd has been found primarily in

the highlands of Costa Rica, and our sampling took place in the lowlands, we sampled for Bd in due to the global importance of this pathogen and its particular relevance for Costa Rican amphibian populations (Daszak et al. 1999; Fisher et al. 2009; Lips 1998).

This study examined assemblages of amphibians living in artificial wetlands in Costa Rica under a suite of pesticide application regimes to determine how these chemicals can affect amphibian community composition, population density, body condition, as well as the prevalence of an important amphibian pathogen, Bd. The objective of this study was to determine if amphibians in this system are showing effects associated with pesticide exposure at any level of organization, to assess the potential for long-term persistence in artificial wetlands. We predicted that body condition, population densities, and species richness would be lower in sites with higher total pesticide application rates and Bd prevalence would be higher, due to pesticides acting as a stressor for amphibians.

Methods

Study Area and Sample Sites

The Rio Tempisque watershed in Guanacaste, Costa Rica is approximately 5,404 km^2 extending from the central mountains to the Pacific coast. Land use in the watershed is a mosaic of urban areas, protected areas, and various agricultural crops, with rice being one of the most abundant.

Sample sites consisted of artificial wetlands in the Rio Tempisque watershed near the town of Cañas (Appendix A). We defined artificial wetlands as any area that is flooded due to human activities, whether permanently, such as a pond, or temporarily, such as in agriculture. We used four rice field habitats as sample sites (Appendix B), due to the perceived importance of rice fields as surrogate amphibian habitat (Duré et al. 2008; Machado and Maltchik 2010). All

sampling took place during the transition from the dry season to the wet season (March to June) in 2010 – 2012, because the beginning of the wet season is the peak breeding period for most local amphibian species.

Sample sites varied in their amount of pesticide application. Pesticide application was estimated using landowner surveys, as explained below. Pesticide exposure varied from none reported to levels typical of commercial rice production. This range of pesticide exposure provided a gradient of exposures in habitats that in all other regards are extremely similar.

Landowner/Manager Surveys

The researchers contacted the landowners or managers of all sample sites personally. Each landowner or manager answered standardized survey questions regarding the types and quantities of pesticides applied (Appendix C). These data (Appendix D) were then used to calculate the total pesticide application rate per hectare per year for each separately managed site.

Soil Pesticide Concentration Analysis

Soil samples were collected in May - July of 2012. Two samples were collected from each field site, approximately five weeks apart. Samples were a composite collected from the top 25 mm of soil at five locations around the field sites, from areas that were moist but not waterlogged. The soils were placed in 75 mL plastic containers and stored at -20 ^{o} C until export to the UGA Agricultural Services Laboratory, Athens, GA. Samples were received at the UGA Agricultural Services Laboratory, assigned individual identification number and stored at – 4^{o} C until analysis. Soil sample composites were soxhlet extracted with ethyl acetate for 4 hrs. The extract was concentrated on rotary evaporator and made to a final volume of 2 ml. for GLC analysis. The extract was then analyzed for nitrogen and phosphorus-containing pesticides using

a Perkin Elmer Autosystem Gas Chromatograph equipped with a NP detector and a ZB-5 Megapore (0.53mm) 30-m column. The column was programmed from 135 to 275^{o} C at 5^{o} C min^{-1}. A fortified sample and reagent blank was included with the sample extraction set. Potential positive residues were quantified by external standardization (3 point calibration) with the lower reporting limit set equal to the lowest calibration standard. The chromatography is a modification of EPA Drinking Water Method 507 adapted to current laboratory analytical systems.

The extract was also analyzed for chlorinated pesticides using a Agilent 7890 series Gas Chromatograph equipped with dual Ni63 electron Capture detectors and a ZB-5 Megapore (0.53mm) 30-m columns. The column was programmed from 135 to 275^{o} C at 5^{o} C min^{-1}. A fortified sample and reagent blank was included with the sample extraction set. Potential positive residues were again quantified by external standardization (3 point calibration) with the lower reporting limit set equal to the lowest calibration standard. The chromatography is a modification of EPA Drinking Water Method 508 adapted to current laboratory analytical systems.

Amphibian Captures and Sample Collection

All procedures were approved by the University of Georgia's Institutional Animal Care and Use Committee (AUP #A2012 03-011). Amphibians were captured during 26 sampling dates for the entire community and 47 total sampling dates for *Rhinella marina* evenly distributed throughout the sampling period. Additional sampling dates for *R. marina* were conducted due to another separate species-specific study. Rice fields were walked and an attempt was made to capture any amphibian that was visualized or heard calling. Once caught, individuals were placed in clean plastic bags, identified to species, weighed to the nearest gram,

and measured to the nearest millimeter. Body condition scores were calculated as weight/snout-to-vent length (SVL) for the six most common species where sufficient sample sizes could be obtained. Because of morphological differences between species, body condition can only be compared within a single species, so each species was analyzed separately. We calculated catch/unit effort for all species, as an estimate of relative population densities among study sites. In addition to body condition and catch/unit effort, we also assessed the Bd infection status of a subset of captured amphibians. The ventral surface of each amphibian was swabbed 20 times using a sterile cotton swab to collect DNA for Bd testing following standard sampling techniques (Hyatt et al. 2007). Swabs were frozen -20˚C dry in sterile microcentrifuge tubes until extraction.

Bd PCR

DNA was extracted from swabs using Qiagen DNeasy kits (Qiagen, Venlo, Limburg, Netherlands) following the manufacturer's directions modified for extractions for swabs, using the methods of M. Yabsley, and the DNA product was tested for Bd by conventional PCR following the methods of (Annis et al. 2004).

Call Sampling

Call sampling was conducted to assess community composition. It was performed at sunset for a 30-minute period during a total of 47 sampling dates. The same researcher conducted all call sampling, to minimize sampling bias. Using previously recorded calls of the approximately 15 species in the region as a reference (provided by S. Connelly), all breeding calls heard during the sampling period were identified to species. The presence and absence data from call sampling was supplemented by visual identifications made during captures.

Statistical Analysis

Data on total pesticide application rate were used in analysis rather than quantitative soil pesticide concentrations, because concentrations in soil samples were below detection limits. Pesticides could not be analyzed individually or by group, as these measures covaried with total application rates. Total pesticide application rates were calculated as total liters of herbicides and insecticides applied per hectare per year. The log of total pesticide application rates was used, due to a right skewed distribution. Body condition was analyzed using general linear mixed-effects models with site as a random effect. Fixed effects included total pesticide application rate, year captured, and day of year captured. A stepwise regression was used to eliminate the variable with the highest p-value until the remaining model included only significant effects (p-value$\leq$0.05). Catch/unit effort was analyzed using a general linear mixed-effects model with total pesticide application rate, year captured, day of year captured, species, and species-pesticide interactions as fixed effects, and site as a random effect. A stepwise regression was used to eliminate nonsignificant variables.

Call sampling was analyzed with Nonmetric Multidimensional Scaling (NMDS). For community analysis, NMDS requires Bray-Curtis dissimilarity values, or some other measurement of distance between communities, for every pairwise comparison of observations (Gardiner et al. 2009) so a Bray-Curtis dissimilarity matrix was created using the number of species detected and species identities on each call sampling night. This matrix was then used to plot the communities in an n-dimensional space, while preserving the distances, or Bray-Curtis dissimilarity values, between points. Forty iterations were conducted to ensure an optimal solution. We began with a two dimensional analysis, and added dimensions until there was not a meaningful change in the plot. We assessed the within site variance in community composition

more closely using the within site Bray-Curtis dissimilarities. These values were analyzed using a one-way ANOVA and a Tukey's Honestly Significant Difference (HSD) test. Bd infection status was not analyzed, as no amphibians were found to be positive for Bd. All analyses were completed using the program R version 3.0.1 with package lme4.

Results

A total of 486 amphibians captured were identified as 11 individual species. Call sampling was completed on 47 nights, and detected an additional two species for a total of 13 (Table 2.1). Body condition was analyzed for 390 amphibians from the six species with the largest capture sample sizes. Catch/unit effort was assessed for the capture of 436 amphibians over 26 nights of community capture and 47 nights of *R. marina* capture.

Body Condition

Of the six species for which body condition scores were analyzed (Table 2.2), total pesticide application rate was a significant predictor for one, *Leptodactylus fragilis* (p-value=0.0475, n=101) (Table 2.3). *L. fragilis* captured at sites with higher pesticide application rates had significantly lower body condition scores than those captured in sites with lower pesticide application rates (Figure 2.1). Year captured was a significant predictor for three species: *Engystomops pustulosus* (p-value=0.01046, n=66), *Lithobates forreri* (p-value=0.03545, n=116), and *R. marina* (p-value<0.00001, n=45). For all three species, body condition scores were significantly higher in 2012 than in 2011. Day of year captured was a significant predictor for *E. pustulosus* and *L. forreri*. For *E. pustulosus*, capture on earlier days of year was associated with higher body condition scores (p-value=0.00127), while for *L. forreri,* capture on later days of year was associated with higher body condition scores (p-values=0.0302). For two species,

Dendropsophus microcephalus (n=21) and *Leptodactylus melanonotus* (n=41), no predictor variable was significant.

Bd Infection Status

Bd infection was not detected in any species (n=90).

Catch/Unit Effort

Catch/unit effort varied widely among species (Table 2.4). Total pesticide application rate was not a significant predictor for community catch/unit effort (n=307). However, the species-pesticide interaction term was significant for *L. melanonotus* (p-value=0.000545) and marginally significant for *R. vaillanti* (p-value=0.064764). For both species, higher pesticide application rates were correlated with lower catch/unit effort (Figure 2.2).

Call Sampling

A total of 13 species were detected, with the number of species found at one site across all sampling nights varying from 9 to 12 (Table 2.5). All sites shared nine core species and the remaining four species were detected at a subset of sites. The NMDS analysis found a two-dimensional solution with minimal stress (<2) (Figure 2.3). The NMDS plot provides a visual approximation of both the within-site community composition variance (among sampling dates) and the among-site community composition variance. The size of the polygon relates to the within-site variance, and the relative position of the polygons relates to the among-site variance. According to this analysis, there was no pattern of among-site community composition related to total pesticide application rates, since all sites overlap substantially. However, the size of the polygons varied among sites, meaning that the within-site community composition variance differed among sites. An ANOVA analyzing the within site Bray-Curtis dissimilarities was significant (p-value<0.00001), so a Tukey's HSD test was conducted. The SB site had

significantly higher within-site variability than all other sites (p-values < 0.0015), and the UT site had significantly higher within-site variability than the LPO site (p-value = 0.0146) (Figure 2.4). In general, within-site variability increased as total pesticide application rate increased.

Discussion

Overall, pesticide application rate appears to act as a stressor for amphibians in this system, but only for some species. High pesticide application rates were related to low body condition scores and relative population densities for some species, and species richness and community composition showed some differences among sites. A relationship with Bd prevalence could not be determined, as Bd DNA was not detected in any individual tested.

Amphibian community composition was similar among all of our sampling sites, regardless of pesticide application rate. However, species richness differed among sites, with a general trend for a higher number of species found in sites with lower total pesticide application rates. Communities were highly nested, with nine core species found in all sites and four additional species only found in a subset of sites. In addition, the similarity of the community through time varied among sites. Sites with higher total pesticide application rates had greater variability in the number of species detected per sampling event. Species lost from sites with high pesticide application rates are most likely more sensitive than the general community to the effects of pesticide exposure, and are therefore unable to maintain viable populations in these habitats (Ferreira and Beja 2013). The lower community similarity through time in sites with high pesticide application rates suggests that some species are using these habitats more ephemerally than sites with lower pesticide application rates. While these rice fields may be important habitat for breeding, lower food availability or lack of other important habitat features

could be necessitating movement of amphibians into other habitats, even during the breeding season.

Community catch/unit effort was not significantly affected by total pesticide application rates. However, two species (*L. melanonotus* and *R. vaillanti*) were found in lower densities at sites with higher total pesticide application rates. Pesticide exposure could result in lower population densities if survival is reduced in these habitats, or if food availability is reduced by insecticide usage. Given that only two species showed differences in population densities, rather than the community overall, we believe that survival is lower for these two species in sites with higher pesticide exposure. Previous work has shown that pesticide exposure can directly result in amphibian mortality (Bernal et al. 2009; Bruhl et al. 2013; Edginton et al. 2004; Mann and Bidwell 1999; Relyea 2004; Relyea 2005), although most of these studies measure short-term survival after acute exposure. However, mesocosm studies have shown that long-term exposure can result in mortality through a chain of indirect effects. For instance, Relyea and Diecks (2008) demonstrated that sublethal concentrations of malathion caused a die-off of zooplankon, resulting in a trophic cascade that led to leopard frog mortality. In addition, several recent modeling studies provide evidence that population densities of amphibians can be reduced by environmental contaminants (Karraker et al. 2008; Salice et al. 2011; Willson et al. 2012).

Total pesticide application rate was a significant predictor of body condition score for only *L. fragilis*. For the other five species analyzed, year captured and day of year captured were significant predictors for *L. forreri* and *E. pustulosus*, and year captured was a significant predictor for *R. marina*. There were no significant predictors for *D. microcephalus* and *L. melanonotus*. Lower body condition scores of *L. fragilis* in habitats with high pesticide application rates are most likely due to either lower availability of prey or a direct effect of

pesticide on metabolism. Several studies have found changes in amphibian lipid or polyamine metabolism as a result of pesticide exposure (de Schroeder and de D'Angelo 2000; Gurushankara et al. 2007; Lascano et al. 2011; Sotomayor et al. 2012). While most studies have been conducted on embryos to assess the effects of these changes on reproductive success, changes in metabolism could affect adult growth and body condition as well. In adult amphibians, Brodeur et al. (2011) found reduced body condition for four amphibian species in areas of intensive agriculture with heavy pesticide use, despite finding pesticide concentrations below detection limits at many sites.

Year captured as a significant predictor for body condition scores is a reflection of stochastic interannual variation in the environment. Because all three species for which year was a significant predictor had higher body condition scores in 2012, this relationship most likely reflects an actual change in habitat quality or food availability for this year. Unfortunately, we could not further analyze this relationship because climate data is not publicly available in Costa Rica. Day of year captured most likely reflects seasonality. *L. forreri* captured on later days of the year had higher body condition scores, while *E. pustulosus* captured on later days of the year had lower body condition scores. This difference may be due in part to different breeding seasons. Since sex and reproductive status were not assessed for these amphibians, both of these metrics probably had an effect on body condition scores overall, and females would be larger during the breeding season. Finally, the two species for which body condition scores had no significant predictors had the lowest sample sizes (*D. microcephalus* n=21, *L. melanonotus* n=41), and this may have limited our power to detect differences.

Bd DNA was not detected in any individuals tested. While Bd is incredibly important for many amphibian communities in Costa Rica (Lips 1998; Lips et al. 2008), it is generally found in

the highlands, in cool moist areas. Our study sites are in the lowlands, with high temperatures and relatively low rainfall, and as such are most likely outside of the environmental range for the pathogen (Fisher et al. 2009; Kilpatrick et al. 2010; Murray et al. 2011). Bd has been found at the lowland site of La Selva Biological Station (Whitfield et al. 2012; Whitfield et al. 2013), but this site is in the lowland wet forest whereas ours are in the lowland dry forest. The differences in precipitation and humidity between these sites are likely very important for the pathogen.

For all metrics, responses varied among species. These differences are most likely attributed to differences in susceptibility to pesticides due to a variety of life history characteristics. Species with large home ranges may only spend a fraction of their time in rice field habitats, and may spend the rest of their time in areas with lower exposure to pesticides. Future studies could quantify movement in and out of rice fields using drift fences to determine if various species are spending different amounts of time in these habitats. Similarly, species that are not entirely dependent on wetland habitats may only come into rice fields to find water for breeding and spend the rest of their time in areas with lower pesticide exposure (Hartel et al. 2011; Jeliazkov et al. 2014). Ovipositing in these fields could result in high selection pressure on eggs and larvae, so that individuals that survive to be adults found in these habitats are resistant to pesticides (Cothran et al. 2013; Hua et al. 2013). In addition, generalist and specialist species may have different population level responses to pesticide exposure. “Weedy” species may be found in all sites, either because pesticides do not affect them or because they are so common that new individuals will continue to colonize the rice fields regardless of negative effects (Ferreira and Beja 2013). High reproductive rates of these species may result in the opportunity for rapid selection for resistance to pesticides. These species may even be at a competitive advantage in habitats with high pesticide exposure, due to the presence of fewer competitors.

However, the loss of sensitive specialist species from these sites would simplify community structure, and in some areas, could leave these species with few other habitat options.

Throughout the tropics, agricultural land use continues to expand (Lacher and Goldstein 1997), and in many areas, rice fields are a dominant landscape feature (Organization for Tropical Studies 2001). Declining natural wetlands have led to the proposal of rice fields as a suitable alternative habitat for amphibians, owing to the superficial similarities of water inundation and large amounts of plant biomass and aquatic species. Machado and Maltchik (2010) compared amphibian diversity in natural wetlands and rice fields in Brazil. While the authors concluded that rice fields could provide an alternative habitat, community composition differed and both the mean richness and mean abundance of amphibian species observed in the rice fields studied were significantly lower than in the control natural wetlands. Another study in Argentina (Duré et al. 2008) found that amphibian diversity varied within different microhabitats in the rice fields, with natural vegetation providing the most diverse habitat. These studies suggest that rice fields are not a complete substitute for the habitat provided by natural wetlands. Indeed, simply finding large numbers and high diversity of amphibians in rice field does not mean that rice fields are good habitats. They may, in fact, be attractive sink habitats where mortality exceeds reproduction (Pulliam 1988). Further study is necessary to determine if amphibian communities are capable of persisting in rice fields for long periods of time, and whether persistence requires an influx of individuals from connected natural habitats (Willson and Hopkins 2013).

If rice fields are expected to be an alternative habitat for amphibians where natural wetlands are scarce, the loss of sensitive species makes this much less tenable. Universally common species are not the ones in need of alternative habitats, and the loss of a subset of species from the community does not preserve community structure. Sensitive species could be

in danger of local extirpation or extinction if they cannot survive in artificial wetlands, particularly if they are not widely distributed. However, rice fields and other artificial wetlands could be managed to improve their ability to be surrogate amphibian habitat. Organic rice farming is currently difficult due to low yields, but is becoming more economically viable. In addition, pesticides known to have high toxicity could be replaced with less toxic alternatives. For instance, in a review of the pesticides used in Costa Rica by Humbert et al. (2007), of the 30 most commonly used chemicals, the majority of aquatic and human toxicity (75% and 90% respectively) were due to a small number of active substances (5, 40% of total and 2, 10% of total respectively). As a result, a large benefit could be achieved by small changes, substituting these particularly harmful chemicals for others with lower toxicity. While pesticides are not the only difference between rice fields and natural wetlands, removing pesticides could remove a significant stressor for the most sensitive species. Future studies could determine if hydroperiod, field size, or other habitat features could also be altered to better support complete amphibian communities (McIntyre et al. 2011; Paton and Crouch 2002), without significantly affecting product yield.

This study provides evidence that pesticide exposure can be an important stressor for some amphibian species, and is associated with changes at the individual, population, and community levels. Given the current status of amphibian declines, management of artificial wetlands as habitat for amphibians should be a priority for conservation efforts. Minimizing pesticide exposure could make these habitats more suitable for a wider range of species, and result in more natural communities that protect sensitive species.

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Table 2.1. Summary of species detected. Life history information is taken from Savage (2002).

Species	Family	Life History Traits	Successful Detection Method(s)
Dendropsophus microcephalus	Hylidae	Habitat: Lowland Moist, Wet, and Dry Forests and Premontane Wet Forest and Rainforest zones. Temporary ponds in open areas, especially secondary growth, pastures, and roadside ditches. Breeding: Throughout the wet season in response to heavy rains.	call sampling and capture
Engystomops pustulosus	Leptodactylidae	Habitat: Lowland Dry, Moist, and Wet Forest and marginally in the Premontane Wet Forest of the Pacific versant. Natural or human-made temporary ponds, puddles, potholes, hoofprints, ditches, pastures, gardens, secondary growth and along forest edges or small permanent ponds or water catchment. Breeding: Early in the wet season (May to June) and sporadically thereafter until November.	call sampling and capture
Incilius coccifer	Bufonidae	Habitat: Lowland Dry Forest, and Premontane Moist Forest, Wet Forest, and Rainforest zones. Pastures, roadside ditches, and gardens and vacant lots in urban areas. Breeding: Mid-May to mid-June, but some reproductive activity occurs into August.	call sampling and capture
Leptodactylus bolivianus	Leptodactylidae	Habitat: Pacific Lowland Dry, Moist, and Wet Forest zones. Near shallow bodies of water, including temporary ponds and roadside ditches. Breeding: Throughout the wet season.	call sampling and capture
Leptodactylus fragilis	Leptodactylidae	Habitat: The lowland forest zones of the Pacific versant and marginally on the northern Atlantic plains. Near marshes, ponds, and any temporary lenthic body of water during rainy periods, and is most common in open and disturbed sites. Breeding: After heavy rains throughout the wet season.	call sampling and capture

Leptodactylus melanonotus	Leptodactylidae	Habitat: Lowland and Premontane Wet Forest zones. Near or in temporary ponds, roadside ditches, swamp margins, and marshes. Breeding: Primarily during the early heavy rains and sporadically thereafter to August.	call sampling and capture
Lithobates forreri	Ranidae	Habitat: Lowland Dry Forest and marginally into the Lowland Wet and Premontane forest zones. Ponds and marshy situations. Breeding: Wet season, frequently in temporary ponds.	call sampling and capture
Lithobates vaillanti	Ranidae	Habitat: Lowland Dry, Moist, and Wet Forests and marginally in Premontane Rainforest. Associated with lentic waters. Breeding: At least from June to August.	call sampling and capture
Rhinella marina	Bufonidae	Habitat: All lowland and premontane zones, and ranging to 2100 m in the lower montane zone. Disturbed areas and around human habitations. Breeding: Opportunistic reproduction occurs sporadically beginning with the first rains of the season, and breeding congresses throughout the rainy season.	call sampling and capture
Rhinophrynus dorsalis	Rhinophrynidae	Habitat: Lowland Dry Forest. Roadside ditches, pastures, cultivated fields and other open areas as well as the forest. Breeding: First heavy rains in late May or early June.	call sampling
Scinax staufferi	Hylidae	Habitat: Primarily in Lowland Dry Forest region, and also in the Lowland Moist Forest in the upper Rio San Juan drainage. Temporary shallow ponds, flooded pastures and roadside ditches. Breeding: Throughout most of the wet season after heavy showers.	call sampling and capture
Smilisca baudinii	Hylidae	Habitat: Commonly in the Lowland Dry Forest areas, but also occurring in Lowland Moist and Wet Forests, and rarely in Premontane Rainforest. Near temporary ponds and flooded fields. Breeding: Large congregations after the first heavy rains (May).	call sampling and capture
Trachycephalus venulosus	Hylidae	Habitat: Lowland Dry, Moist, and Wet Forests. Near temporary ponds. Breeding: Explosive breeder after heavy rains.	call sampling

Table 2.2. Summary statistics for body condition data.

Species	Average	Standard Deviation	Minimum	Maximum
Dendropsophus microcephalus	0.048	0.014	0.015	0.076
Engystomops pustulosus	0.058	0.021	0.022	0.134
Leptodactylus fragilis	0.094	0.049	0.024	0.207
Leptodactylus melanonotus	0.119	0.046	0.025	0.236
Lithobates forreri	0.274	0.257	0.026	1.009
Rhinella marina	1.094	0.347	0.550	2.105

Table 2.3. Summary of body condition models. Plus and minus signs describe the direction of the significant relationship. For example, a plus sign at the intersection of *Lithobates forreri* and Day reflects that higher body condition scores for *Lithobates forreri* are associated with capture on later days of the year. For year, the year given matches the direction of the relationship. For instance, "+ 2012" means that the response variable was significantly higher in 2012. "None" describes no significant relationship.

Species	Pesticide	Year	Day
Dendropsophus microcephalus	none	none	none
Engystomops pustulosus	none	+ 2012	-
Leptodactylus fragilis	-	none	none
Leptodactylus melanonotus	none	none	none
Lithobates forreri	none	+ 2012	+
Rhinella marina	none	+ 2012	none

Table 2.4. Summary statistics for catch/unit effort data.

Species	Average	Standard Deviation	Minimum	Maximum
Dendropsophus microcephalus	0.808	1.443	0	5
Engystomops pustulosus	2.538	2.420	0	8
Incilius coccifer	0.192	0.801	0	4
Leptodactylus bolivianus	0.115	0.588	0	3
Leptodactylus fragilis	3.885	5.901	0	20
Leptodactylus melanonotus	1.577	2.469	0	9
Lithobates forreri	4.462	3.669	0	15
Lithobates vaillanti	0.154	0.464	0	2
Rhinella marina	1.277	1.885	0	6
Scinax staufferi	0.077	0.392	0	2
Smilisca baudinii	0.654	1.355	0	5

Table 2.5. Number of species detected by call sampling at each site.

Site	Total Pesticide Application Rate (L/ha/yr)	Number of Species Detected
SB	22.6	9
LPC	6.57	11
UT	3.97	12
LPO	0.200	10

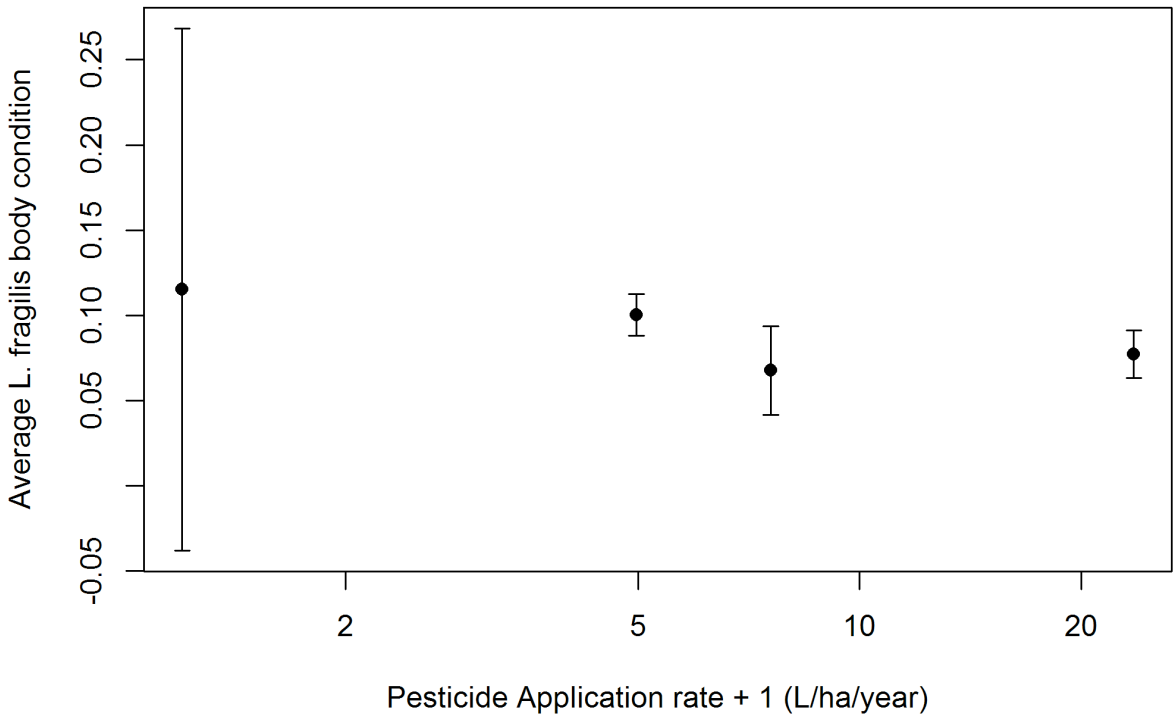

Figure 2.1. Average body condition for *Leptodactylus fragilis* along a gradient of total pesticide application rates. Errors bars represent 95% confidence intervals. Sites with higher total pesticide application rates had significantly lower *L. fragilis* body condition (p=0.0475).

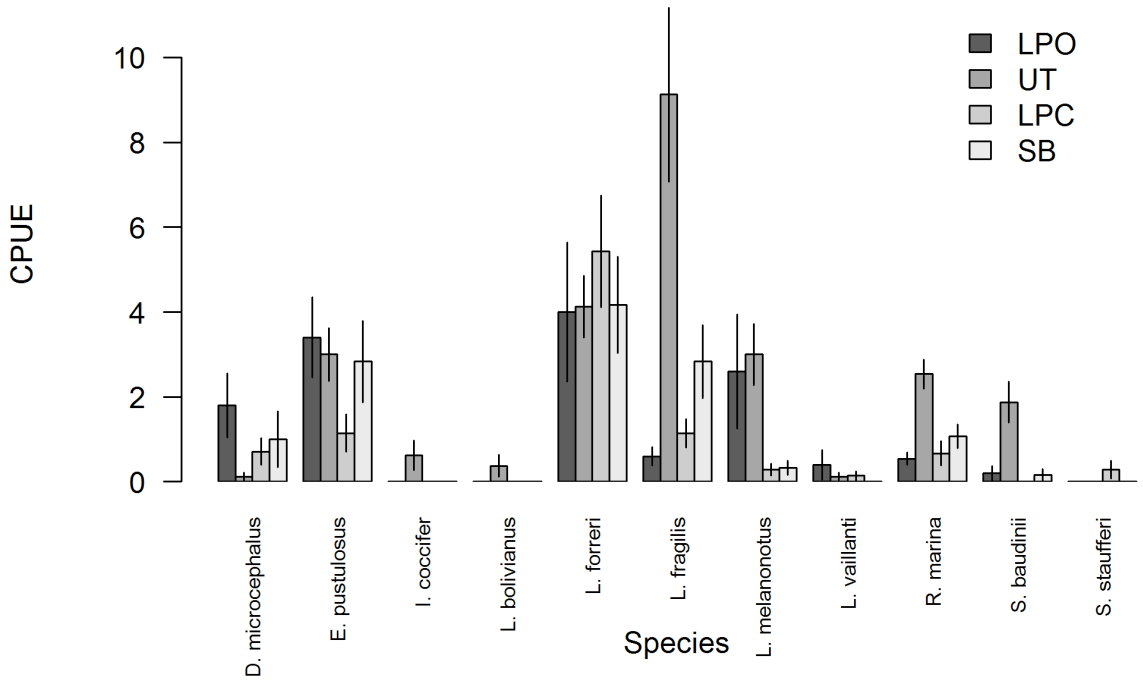

Figure 2.2. Average catch/unit effort for 11 species across four sites along a gradient of total pesticide application rates. Error bars are 95% confidence intervals. Higher catch/unit effort for *L. melanonotus* (p-value=0.000545) and *L. vaillanti* (p-value=0.064764) were associated with lower total pesticide application rates.

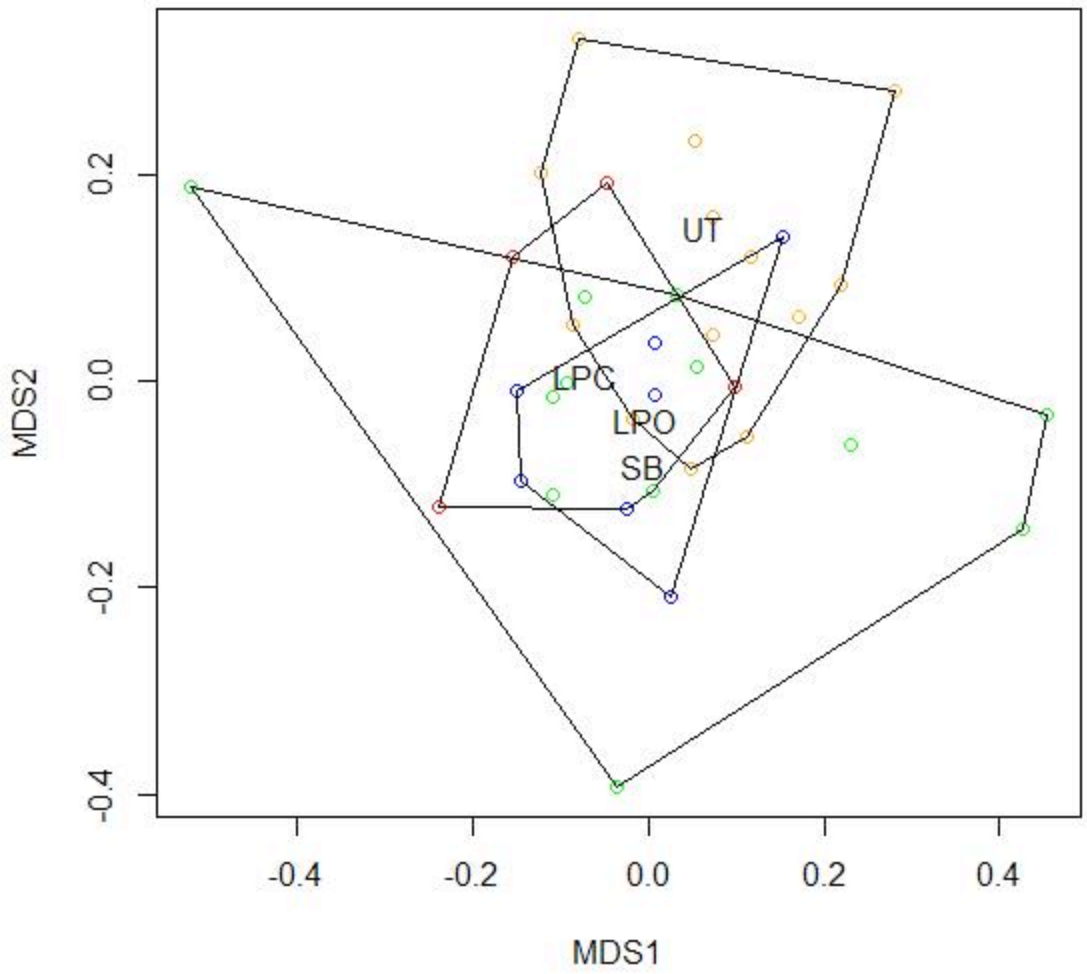

Figure 2.3. Nonmetric multidimensional scaling (NMDS) ordination plot of call sampling results. Polygons bound all community composition observations for a site. Larger polygons correspond with greater within site differences in community composition among sampling dates. Relative location of polygon with regard to other polygons is determined by similarities in community composition among sites.

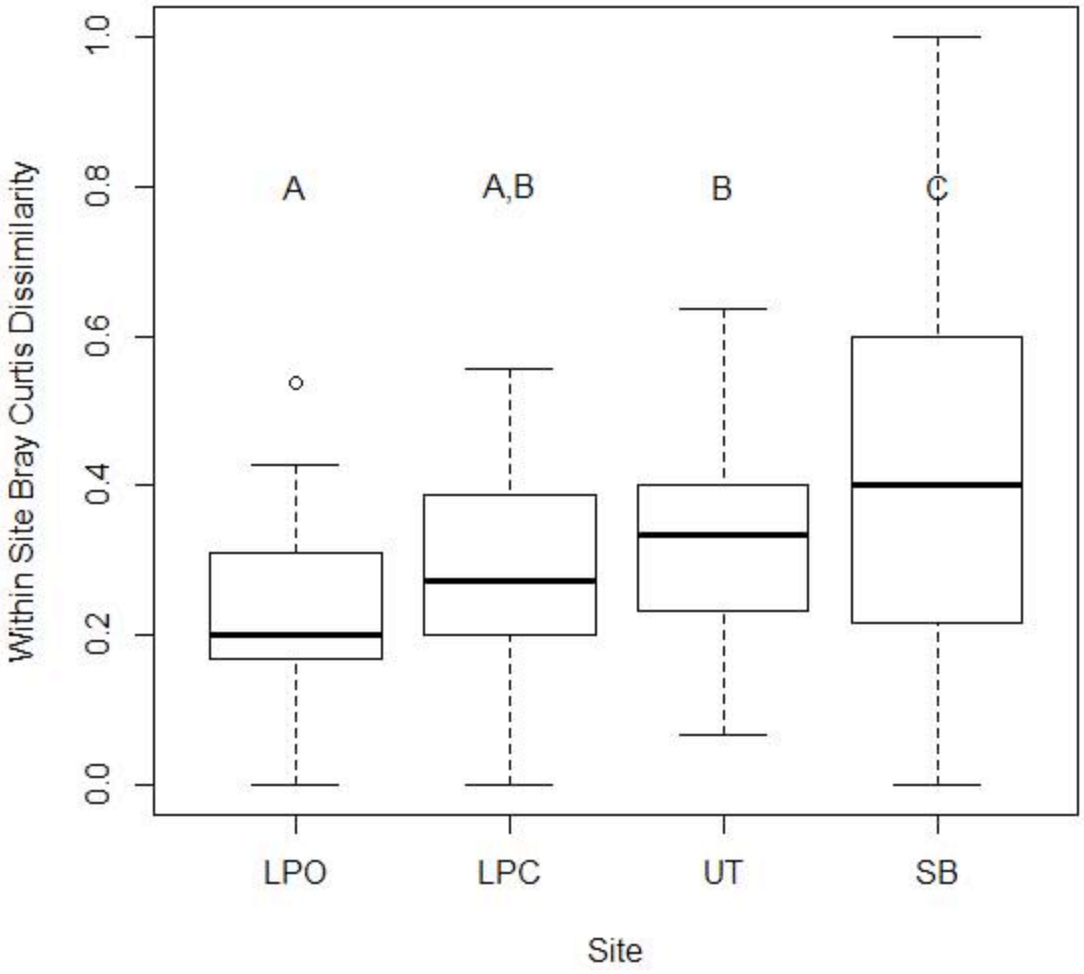

Figure 2.4. Boxplot of within site Bray-Curtis dissimilarity scores. Letters above boxplots denote statistical significance based on ANOVA and Tukey's HSD test. In general, within site dissimilarities increased with increased total pesticide application rate.

CHAPTER 3

RELATIONSHIP OF PESTICIDES AND RHINELLA MARINA STRESS, IMMUNE FUNCTION AND CONDITION[2]

[2]McDowell, K. M. S., S. M. Hernandez, K. Navara, A. Ellis, and C. R. Carroll. To be submitted to Science of the Total Environment.

Abstract

The rapid decline of wetlands results in competitive advantage to amphibian species in many areas that can adapt to utilize alternative habitats. However, use of human-modified habitats can result in exposure to a variety of anthropogenic chemicals, including pesticides. We used the cane toad, *Rhinella marina*, as a model species to assess the importance of pesticide exposure for amphibians in artificial wetland habitats in Guanacaste, Costa Rica. We measured a variety of physiological indicators, such as body condition metrics, corticosterone levels, and immune function from toads collected from sites with a variety of pesticide application rates. Our objective was to determine if the values of these indicators were related to pesticide exposure, which could potentially lead to declines in fitness. Pesticide application rate was a significant predictor of the presence of insect head capsules in toad gut contents indicating adaptive foraging, an important component of fitness. Other variables showed no significant relationship with pesticide application rate. Contrary to expectations, *Rhinella marina* does not appear to be immunosuppressed by pesticide exposure. Interestingly, the higher presence of insect head capsules in gut contents from toads in sites with higher pesticide application rates suggests that insecticide use may not negatively impact food availability for toads in these habitats. This result may not hold true for other amphibian species, however; due to the cane toad's particularly indiscriminate feeding habits, they may be able to prey switch to insect taxa less affected by insecticides. However, there were no ovulating females found in sites with higher pesticide application rates, and intersex individuals were relatively common among all sites. This result suggests that the reproductive function of amphibians in these habitats could be compromised. The immune function and body condition of more sensitive amphibian species might have a

greater association with pesticide exposure than that of the cane toad, and this likely difference among species requires further research.

Introduction

The alteration of natural ecosystems for human use has caused drastic changes in the habitats of many organisms. However, not all organisms have the same sensitivity to human caused disturbance. A variety of organisms are capable of surviving, and even thriving, in human dominated systems. However, using human impacted habitats exposes these organisms to all of the anthropogenic chemical inputs in these systems, including pesticides (Linzey et al. 2003; Mann et al. 2009; McCoy et al. 2008). This scenario is a particular issue for wetland habitats. The rapid decline of natural wetlands (Millenium Ecosystem Assessment 2005; Prigent et al. 2012) has led to many species, especially amphibians, relying on artificial wetlands and other human impaired environments (Duré et al. 2008; Machado and Maltchik 2010). Despite their persistent presence in impaired systems, organisms still can be susceptible to non-target effects of pesticides.

Pesticides have been shown to cause a myriad of effects in amphibians and other organisms, from increased pathogen and parasite susceptibility (Forson and Storfer 2006; Kelehear et al. 2009), to indirect effects through trophic cascades (Relyea and Diecks 2008), to reproductive problems (Hayes et al. 2003; McCoy et al. 2008), and death (Davidson and Knapp 2007; Mann et al. 2009). Laboratory studies have demonstrated that pesticides can alter multiple aspects of amphibian immune function (Christin et al. 2003; Christin et al. 2004; Gilbertson et al. 2003; Hayes et al. 2006), and alter an individual's susceptibility to specific parasites (Forson and Storfer 2006; Kerby and Storfer 2009; Rohr et al. 2008). Pesticides can also affect growth and reproduction, thereby altering condition (Baker et al. 2013; Hayes et al. 2003; Hayes et al. 2006; McCoy et al. 2008; Relyea 2004).For all of these reasons, pesticides may be a stressor for amphibians which can cause physiological changes.

Several measures can be used to estimate this physiologic stress. Corticosterone (CORT) is the primary stress hormone for amphibians. In herpetofauna, the hormone corticosterone can be measured using either blood or fecal samples and has been shown to correlate with body condition and survival probabilities (Romero and Wikelski 2001; Waye and Mason 2008), and chronically elevated baseline CORT can result in immunosuppression (Busch and Hayward 2009). Phytohemagglutinin (PHA) is a plant lectin sometimes used to test the magnitude of immune response to a novel antigen (Brown et al. 2011). When introduced to animal cells, PHA acts as a mitogen (a chemical that triggers mitosis) and stimulates T-cell proliferation (Martin et al. 2006; Siva-Jothy et al. 2001). T-cells are a major component of the early stage of the acquired vertebrate immune response, and their proliferation therefore provides a measure of immunocompetence (Siva-Jothy et al. 2001). The level of response is judged by the amount of inflammation resulting from the introduction of the antigen, generally by injection.

Body condition is a proxy for energy stores, and has been used to estimate the effects of a variety of stressors (Bancila et al. 2010; MacCracken 2005; MacCracken and Stebbings 2012; Reading 2007; Waye and Mason 2008). In addition to the traditional calculated body condition score, toad fat bodies were also directly weighed and the presence or absence of insect head capsules in gut contents was scored. *R. marina* offers another measure of condition in its parotid glands. These glands contain large quantities of nitrogen-based complex toxins that are physiologically costly to produce. Gland size is thought to be related to toxin content (Phillip and Shine 2005); therefore toads that are in poor condition may have smaller parotid glands. Lastly, the righting reflex is often used as a method of evaluating neurologic function in amphibians, and the loss of righting reflex is understood to be a sign of extreme stress, lethargy, or neurologic dysfunction (Bennett and Mehler 2006).

Understanding the impacts of pesticides on disturbance-tolerant species provides a conservative estimate of the effects of pesticide exposure to free-living amphibian populations. This makes *Rhinella marina* (*Bufo marinus*, *Chaunus marinus*), commonly known as the cane toad, an ideal model organism, as it has been shown to thrive in human disturbed habitats (Zug and Zug 1979). Cane toads may be expected to show similar morphological signs of stress as other amphibian species. McCoy et al. (2008) demonstrated that cane toads in agricultural habitats have gonadal abnormalities and altered sex hormone profiles and secondary sex characteristics. Their response may be more attenuated than that of other amphibian spcies, due to their affinity for disturbed environments (Rejmanek and Richardson 1996; Zug and Zug 1979) and their success as a widespread invader (Estoup et al. 2001; Phillips et al. 2006; Slade and Moritz 1998). The cane toad is native to Central America, where it has not been well studied. What little is known of the natural history of this species in its native habitat stems from one study conducted in Panama (Zug and Zug 1979). Populations are typically composed of 50 to 150 individuals per hectare in semi-natural habitats. They are indiscriminate feeders, but ants and beetles make up the majority of their diet. They forage in an average of 160 sq m and are very mobile. They are most active in the early evening, and gorge themselves for one night then burrow for several days before emerging to feed again (Zug and Zug 1979). In part due to their inclination toward disturbed habitats, cane toads are frequently found in artificial wetlands in Costa Rica.

In the Guanacaste province of Costa Rica, pesticides are used in a variety of agricultural applications, including rice fields. If concentrations of toxic pollutants from these or other sources are high enough, toad populations may show some physiological effects. To determine how exposure to agrochemicals in artificial wetlands may impact amphibians, we used cane

toads living in artificial wetlands in the Rio Tempisque basin as model organisms to examine how pesticide exposure is related to stress, immune system function, and body condition. The objective of this study was to assess these cane toads for evidence of reduced fitness that could lead to population level effects over time. We expected that cane toads would have lower body condition, lower immune function, and higher corticosterone levels in sites with higher total pesticide application rates. The combination of evaluation of several body condition indices and stress and immune system function assays should provide a robust mechanism for assessing amphibian health in field systems.

Methods

Study Area and Sample Sites

The Rio Tempisque watershed in Guanacaste, Costa Rica is approximately 5,404 sq km extending from the central mountains to the Pacific coast. Land use in the watershed is a mosaic of urban areas, protected areas, and various agricultural crops, with rice being one of the most abundant. Sample sites consisted of artificial wetlands in the Rio Tempisque watershed near the town of Cañas (Appendix A). We defined artificial wetlands as any area that is flooded due to human activities, whether permanently, such as a pond, or temporarily, such as in agriculture. Artificial wetlands used as sample sites included four rice fields, a seasonally flooded woodlot, and an artificial pond and surrounding lawn on a hotel grounds (Appendix B). All sampling took place during the transition from the dry season to the wet season (March to June) in 2010 - 2012, because the beginning of the wet season is the peak breeding period for most local amphibian species.

Sample sites varied in their amount of pesticide application. Pesticide application was estimated using landowner surveys, as explained below. Pesticide exposure varied from none

reported to levels typical of commercial rice production. This range of pesticide exposure provided a gradient of exposures in habitats that in all other regards are extremely similar.

Landowner/Manager Surveys

The researchers contacted the landowners or managers of all sample sites personally. Each landowner or manager answered standardized survey questions regarding the types and quantities of pesticides applied (Appendix C). These data (Appendix D) were then used to calculate the total pesticide application rate per hectare per year for each separately managed site.

Soil Pesticide Concentration Analysis

Soil samples were collected in May - July of 2012. Two samples were collected from each field site, approximately five weeks apart. Samples were a composite collected from the top 25 mm of soil at five locations around the field sites, from areas that were moist but not waterlogged. The soils were placed in 75 mL plastic containers and stored at -20 ^{o} C until export to the UGA Agricultural Services Laboratory, Athens, GA. Samples were received at the UGA Agricultural Services Laboratory, assigned individual identification number and stored at – 4^{o} C until analysis. Soil sample composites were soxhlet extracted with ethyl acetate for 4 hrs. The extract was concentrated on rotary evaporator and made to a final volume of 2 ml. for GLC analysis. The extract was then analyzed for nitrogen and phosphorus-containing pesticides using a Perkin Elmer Autosystem Gas Chromatograph equipped with a NP detector and a ZB-5 Megapore (0.53mm) 30-m column. The column was programmed from 135 to 275^{o} C at 5^{o} C min^{-1}. A fortified sample and reagent blank was included with the sample extraction set. Potential positive residues were quantified by external standardization (3 point calibration) with the lower reporting limit set equal to the lowest calibration standard. The chromatography is a

modification of EPA Drinking Water Method 507 adapted to current laboratory analytical systems.

The extract was also analyzed for chlorinated pesticides using a Agilent 7890 series Gas Chromatograph equipped with dual Ni63 electron Capture detectors and a ZB-5 Megapore (0.53mm) 30-m columns. The column was programmed from 135 to 275^{o} C at 5^{o} C min^{-1}. A fortified sample and reagent blank was included with the sample extraction set. Potential positive residues were again quantified by external standardization (3 point calibration) with the lower reporting limit set equal to the lowest calibration standard. The chromatography is a modification of EPA Drinking Water Method 508 adapted to current laboratory analytical systems.

Amphibian Captures and Sample Collection

All procedures were approved by the University of Georgia's Institutional Animal Care and Use Committee (AUP #A2012 03-011). *R. marina* were captured during 62 sampling dates evenly distributed throughout the sampling period. Toads were held in individual enclosures for 12-36 hours before necropsy, during which time pre-mortem samples were collected. Toads were humanely euthanized following approved methodology for amphibians (American Veterinary Medical Association 2007). Briefly, toads were immersed in a solution containing an overdose of buffered tricaine methanesulfonate (MS-222), followed by cervical pithing, and a complete gross necropsy.

Corticosterone Analysis

Blood for corticosterone analysis was collected immediately upon capture from the abdominal midline vein for a baseline CORT measurement. All blood samples were collected within three minutes of capture; if this was not possible, no blood sample was analyzed for that

individual to avoid measuring an increase in corticosterone level associated with capture stress (Busch and Hayward 2009; Romero and Wikelski 2001). Corticosterone was extracted from plasma samples with an ether extraction. Briefly, 1800 cpm of tritiated corticosterone was added to each sample for later recovery calculation. Next, 3 mL of diethyl ether was added to each sample, the mixture was vortexed for 30 seconds and allowed to settle for 20 minutes. Samples were then snap frozen and the supernatant was poured off and dried using an N_2 stream. Corticosterone was quantified using standard competitive binding radioimmunoassays (using Anti-CORT from MP Biomedicals, Solon, OH) as described in Wingfield and Farner (1975). All samples were analyzed in one assay for each hormone. Average recovery was 85% and intrassay variation was 4.71% for 2011 samples, and 70% average recovery and 2.16% intrassay variation for 2012 samples.

The Righting Reflex

As an additional metric of health, we performed a righting reflex test. The righting reflex is often used as a method of evaluating overall mentation, central nervous function, musculoskeletal strength and vestibular function in amphibians, with the loss of a righting reflex understood to be a sign of extreme stress, lethargy or neurologic dysfunction (Bennett and Mehler 2006). The righting reflex test was conducted following an 18-hour acclimation period after capture. The toad was turned onto its dorsal side on a hard counter, and a timed trial began. The toad was allowed to right itself and then was immediately turned over again. This procedure continued until the toad could no longer right itself. If the toad could not right itself within one minute after being turned over, the trial was concluded. Both the total time of the test and the number of times the toad righted itself were recorded, and each variable was analyzed separately.

PHA Test

The thickness of the second toe web on the right hind foot was measured with digital calipers to the nearest thousandth of a millimeter. The toe web was then injected with 50 uL of 2mg/mL PHA dissolved in phosphate buffered saline (PBS). Injections were made with 27 gauge tuberculin syringes to minimize inflammation due to injection. Toe web thickness was measured at 6, 12, and 24 hrs post injection to monitor inflammatory response. All measurements were made three times and averaged for improved accuracy, and the average pre-injection toe web thickness was subtracted from this value to provide an average inflammatory response for each time point. Only the 12-hr time point was analyzed, because this point was the height of the inflammatory response. To determine the inflammatory effects of PHA at the cellular level, a four mm sample of the toe web where PHA was injected was collected during necropsy and preserved in 10% buffered formalin. Toe web sections were histologically examined and scored for inflammation by a board-certified veterinary pathologist with expertise in herpetofauna. The following scoring system was used: 0 = no significant lesion observed; 1 = edema and/or hemorrhage; 2 = cellular infiltrates.

Body Condition Measurements

Body condition assessment was made using several different methods. Each individual was weighed to the nearest gram and its snout-vent length (SVL) recorded to the nearest millimeter. Body condition scores were calculated as weight/SVL. The longest axis and the widest perpendicular axis of the parotid gland were measured to calculate its area. This area was then adjusted for the size of the toad by dividing by SVL. One gland was measured for each toad. Finally, we weighed the discrete intra-abdominal fat bodies, which provide a more direct way to measure the condition of an amphibian, since a body condition score could be skewed by the

reproductive status of a female or the time since the toad's last meal. Fat bodies were collected and individually weighed to the nearest tenth of a gram as part of the gross necropsy.

Gut Content Analysis

Fecal samples were collected from the terminal section of the gastrointestinal tract during necropsy and preserved in 2.5% potassium dichromate. Samples were sorted for insect head capsules under a dissecting microscope. Gut contents were analyzed as presence/absence of any head capsules, to avoid inaccurate counts due to deterioration.

Statistical Analysis

Total pesticide application rate was used to analyze data rather than quantitative soil pesticide concentrations, because concentrations in soil samples were below detection limits. Pesticides could not be analyzed individually or by group, as these measures covaried with total application rates. Total pesticide application rates were calculated as total liters of herbicides and insecticides applied per hectare per year. The log of total pesticide application rates was used, due to a right skewed distribution. Data were analyzed using general linear mixed-effects models with site as a random effect. Fixed effects varied depending on the response variable, but total pesticide application rate, year captured, day of year captured, and sex of individual were always included. The only exceptions to this were that year captured was not included for either of the righting reflex variables, because this test was only performed on toads captured in 2012, and day of year captured was not included for toe web histopathology scores, due to constraints of the ordinal model. Two sets of models were run for each response variable, one including only the fixed effects mentioned above (referred to as basic model), and one including other effects that could have biological relationships with the response variable (referred to as full model). Additional predictive variables included other measured variables discussed in this paper. The

number of samples per predictive variable was always ≥10, to avoid overparameterization of the models (Vittinghoff and McCulloch 2007). A Pearson Correlation matrix was completed to ensure that auto-correlated variables were not included as effects in the same model. For each set of models, a stepwise regression was employed to eliminate the variable with the highest p-value until the remaining model contained only significant effects (p-value≤0.05). However, marginally significant p-values (≤0.1) for total pesticide application rate are reported. Generally, linear regressions were used, but a logistic regression was used for gut contents analysis, and an ordinal logistic regression was used for toe web histopathology analysis. All analyses were completed using the program R version 2.14.1 with package lme4, except the toe web histopathology analysis, which was completed with package ordinal.

Results

A total of 100 *R. marina* were captured. The number of toads varied for which each response variable was measured (Table 3.1). As a result, the sample size in the full models sometimes became larger during the stepwise regression as predictor variables with smaller sample sizes than the response variable were removed. Predictor variables with much smaller sample sizes than the response variable were excluded whenever possible; however, variables expected to have a strong biological relationship were included in the full model regardless of sample size. The results of both the basic and full models are reported here to avoid losing the power of the full sample size collected (Tables 3.2, 3.3). In cases where the results of the basic and full models differ, this is due to the process of the stepwise regression. Using both sets of models allows us to examine the importance of a variety of predictor variables without losing the emphasis on our predictor variables of primary interest.

Corticosterone

In both models, year captured and sex were the only significant predictors of CORT (p-values=0.0253, 0.0246, respectively, n=59). Toads captured in 2012 had significantly lower baseline CORT than toads caught in 2011. Male toads had significantly higher baseline CORT than females.

The Righting Reflex

The day of year captured and sex were the only significant predictor variables for the total time of the righting reflex test in the basic model (p-values=0.00424, <0.00001, respectively, n=32), although total pesticide application rate was marginally significant (p-value=0.06801). Higher pesticide application rates and males were associated with a longer total test time. A full model was not run for either righting reflex variable, to avoid overfitting a model with small sample size. For number of turns completed during the righting reflex test, sex was the only significant predictor in the basic model (p-value=0.00829, n=32). Male toads completed a significantly higher number of turns than females.

Inflammatory Response to PHA

In the basic model for the 12-hour post injection inflammatory response to PHA, year captured was a significant predictor (p-value=0.0405, n=66). In the full model, year captured and body condition were both significant predictors (p-values=0.00608, 0.04593, respectively, n=66). The response to PHA was greater in 2011 than in 2012, and toads with higher body condition scores had a higher 12-hour PHA response. There were no significant predictors of the toe web histopathology scores (n=56).

Body Condition Scores

Year captured and day of year captured were the significant predictors for body condition scores in the basic model (p-values=0.001, 0.00378, respectively, n=84). In the full model, year captured (p-value<0.00001), the 12-hour inflammatory response to PHA (p-value<0.00001), and fat body weights (p-value<0.00001) were significant predictors (n=64). Toads captured on later days of the year had higher body condition scores than toads captured on earlier days. Toads captured in 2012 had a significantly higher body condition score than toads caught in 2011. Toads with higher fat body scores and higher 12-hour PHA inflammatory response had higher body condition scores.

Fat Body Weights

In the basic model for fat body weights, day of year captured and sex were significant predictors (p-values<0.00001, =0.0413, respectively, n=82). However, in the full model, only day of year captured was a significant predictor (p-value<0.00001, n=82). Toads captured on later days of the year had higher fat body weights than toads captured on earlier days. Female toads had significantly higher fat body weights than males.

Parotid Glands

Year captured was the only significant predictor of parotid gland size in both the basic and full models (n=100). Toads captured in 2011 had a significantly smaller relative parotid gland area than toads caught in 2010 or 2012 (p-value=0.0119).

Gut Contents

Day of year captured was the only significant predictor of gut contents in the basic model (p-value=0.0444, n=80), although total pesticide application rate was marginally significant (p-value=0.0759) (Figure 3.1). Toads captured on earlier days of the year were significantly more

likely to have insect head capsules in their gut contents than toads captured on later days. In the full model, total pesticide application rate was the only significant predictor (p-value=0.0225, n=80). In both models, higher pesticide application rates were associated with a higher probability of the presence of gut contents.

Discussion

We found little evidence that the stress, immune function, and body condition of *R. marina* were associated with pesticide exposure in our artificial wetland sites. Higher total pesticide application rate was associated with higher righting reflex time, although this was a marginal relationship, and a higher probability of presence of insect head capsules in gut contents. Higher righting reflex time reflects a lower performance on this test, in that toads are simply taking longer to complete turns. Interestingly, the higher probability of the presence of gut contents in toads captured in habitats with higher total pesticide application rates suggest that insecticide use does not lead to reduced food availability for toads. Due to *R. marina*'s indiscriminate feeding (Zug and Zug 1979), they may be able to switch to insect taxa less affected by insecticides used in these habitats. Therefore, this pattern may not hold true for other amphibian species with a narrower prey range.

While we were unable to analyze it statistically, we observed a difference in reproductive status in female toads among our study sites. Despite capturing toads during the breeding season, many females were not in breeding condition, and in fact, the only females displaying maturation of the ovaries were in sites with relatively low total pesticide application rates. From a total sample size of 44 female toads, only seven had developed mature ovarian follicles. In addition to this, histologically we identified seven intersex individuals, with both testicular and ovarian tissue. While, these individuals were evenly spread among study sites, we believe it is

noteworthy that this represents 7% of the total individuals sampled. Many studies have found that pesticide exposure can alter amphibian reproduction function. Atrazine has been associated with increases in hermaphroditism (Hayes et al. 2003) and can affect sexual differentiation (Tavera-Mendoza et al. 2002; Tavera-Mendoza et al. 2002). McCoy et al. (2008) found both increased feminization and increased intersex characteristics in *R. marina* in sites with a high percentage of agricultural land use. Further research should ascertain whether *R. marina* and other amphibian species display differential reproductive success in habitats with high pesticide exposure, as this could affect the long-term survival of these populations.

For all other variables, total pesticide application rate was not a significant predictor. While previous studies have found that pesticide exposure can affect amphibian immune function, these studies have primarily been conducted in laboratory settings (Christin et al. 2003; Christin et al. 2004; Forson and Storfer 2006; Hayes et al. 2006; but see Gilbertson et al. 2003; Rohr et al. 2008). It is likely that in these complex natural environments, the effects of pesticide exposure are dampened or offset by other factors. For example, a series of studies on the effects of a glyphosate formulation and pH on amphibian survival used a hierarchical approach, starting with laboratory studies and working up to a field study. While both laboratory and mesocosm studies found significant mortality associated with exposure to the herbicide, the field study found no difference in mortality of amphibians among wetlands in a variety of exposure scenarios, most likely due to the effect of vegetation as a buffer (Chen et al. 2004; Edginton et al. 2004; Thompson et al. 2004; Wojtaszek et al. 2004). Studies such as these suggest that the inherent complexity of ecosystems can at least partially protect amphibians from the potential effects of pesticide exposure, resulting in fewer measurable effects in field studies.

Our study organism, the cane toad *R. marina,* is an abundant and relatively large amphibian, more readily observed and captured than many other amphibians. These traits are all useful characteristics for obtaining relevant permits, capturing, and collecting both pre- and post-mortem biological samples. However, we also chose the cane toad because we believed it would provide us with a conservative estimate of the importance of pesticide exposure for amphibians in artificial wetlands. Due to their generalist nature, ability to survive in human modified systems, and success as a widespread invader (Estoup et al. 2001; Phillips et al. 2006; Slade and Moritz 1998), we expected that *R. marina* might be less sensitive to pesticide exposure than some other amphibian species in the system. Yet, based on our results, it is possible that the cane toad is too conservative a choice, and might not provide a realistic picture of how other amphibians are affected by pesticide exposure in these habitats. Further research is necessary to determine the status of the broader amphibian community, and whether pesticide exposure is a significant stressor for other species.

Year captured was a significant predictor for parotid gland relative area, body condition score, PHA, and baseline CORT. Toads caught in 2011 had higher baseline CORT, higher 12-hour PHA inflammatory response, and smaller parotid gland relative area, while toads caught in 2012 had higher body condition scores. Year captured as a significant predictor reflects stochastic interannual variation. Higher baseline CORT and smaller parotid glands in 2011 may suggest that 2011 was not a good year for toads, although higher PHA inflammatory response in 2011 suggests that toads were still able to mount a cell-mediated immune response. Unfortunately, there is no climate data publicly available in Costa Rica that would allow us to explore this relationship. Day of year captured was a significant predictor for body condition scores, fat body weights, gut contents, and righting reflex time. Toads captured on later days of

the year had higher body condition scores and fat body weights, and shorter righting reflex trial times, but a lower probability of the presence of gut contents. Day of year as a significant predictor reflects seasonality. Early sampling days took place during the end of the dry season, while later sampling days were during the beginning of the wet season. As the wet season progresses, toads may be able to put on more weight, due to increased time spent foraging (Zug and Zug 1979).

Sex was a significant predictor for fat body weights, baseline CORT, righting reflex time and number of turns. Male toads had higher baseline CORT, lower fat body weights, higher number of turns in the righting reflex test, and longer righting reflex times. Males appear to be more stressed and have lower fat body weights, however they also performed better in the righting reflex test. It is possible that the righting reflex test is not an accurate indicator of stress and fitness for this species, which may explain the apparent contradiction here.

Higher body condition scores were associated with higher fat body weights and higher 12-hour PHA inflammatory response. Body condition scores should be positively related to fat body size, so this relationship is expected and shows that our body condition metric is an accurate reflection of condition. Fat body measurements also provide a more direct measurement of energy stores (Waye and Mason 2008), in the case that body condition was obscured by reproductive status or other factors. Body condition has been shown to correlate positively with overall health in amphibians and reptiles (Bancila et al. 2010; Waye and Mason 2008). Generally, organisms in a stressful environment have difficulty maintaining a healthy body condition. Higher PHA inflammatory response associated with higher body condition scores may seem surprising, since a tradeoff between energy investment in immune function and growth is often expected (Lee 2006; Phillips et al. 2010). However, because body condition is not really a

metric of growth, but rather overall condition, it is likely that toads that are able to invest in immune function are able to invest more across the board. There is evidence that PHA inflammatory response is an accurate indicator of fitness, and that individuals with high body condition generally have a greater PHA inflammatory response (Martin et al. 2006).

Our study would have benefited from quantifying pesticide concentrations at our study sites. The pesticide residues in our soil samples were below the detection limits, and thus we were unable to use quantitative concentrations for our analyses. Soil samples were collected during amphibian fieldwork, and were not tied to timing of pesticide applications in any way, which may have limited our ability to detect pesticide residues. Although toads were necropsied, tissue samples were not analyzed for pesticide concentrations. Fat bodies are the most appropriate tissue to analyze for some pesticides, and for many toads, the fat bodies were extremely small or nonexistent. Because the distribution of toads with no fat bodies was uneven among our sample sites, it was impossible to perform an unbiased analysis of pesticide concentrations in toad fat bodies. Future studies should attempt to quantify pesticide concentrations in amphibians to more tightly correlate individual exposure with physiological response variables.

The variables we measured for stress, immune function, and body condition of R. marina were not significantly associated with total pesticide application rates in our artificial wetland study sites, with the exception of presence of insect head capsules in gut contents and righting reflex test time. These results suggest that these habitats may be sufficient surrogate habitat for at least some amphibian species. However, our results should be viewed with caution when applied to other, more sensitive species, as they may be affected differently, and impacts should be assessed at the community level.

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Table 3.1. Summary statistics for response variables.

Response Variable	Average	Standard Deviation	Minimum	Maximum	Number of Toads Measured
Baseline CORT	2.072	2.237	0	9.730	59
Righting Reflex Time	20.281	12.208	10	61	32
Righting Reflex Turns	2.875	2.791	0	12	32
12-hour PHA Response	0.120	0.158	-0.179	0.943	66
Body Condition Score	1.222	0.440	0.500	2.684	84
Fat Body Weight	2.223	4.016	0	22.5	82
Parotid Gland Area	8.942	2.254	3.051	14.244	100

Table 3.2. Summary of basic mixed models. Plus and minus signs describe the direction of the significant relationship. For example, a plus sign at the intersection of Body Condition Score and Day reflects that higher body condition scores are associated with capture on later days of the year. Where necessary for categorical variables, the category given matches the direction of the relationship. For instance, "+ 2012" means that the response variable was significantly higher in 2012. "None" describes no significant relationship. "N/A" references variables that were not included in the model. Marginally significant relationships are denoted with "(marginal)".

Response Variable	Pesticide	Year	Day	Sex
Baseline CORT	none	+ 2011	none	+ M
Righting Reflex Time	+ (marginal)	n/a	-	+ M
Righting Reflex Turns	none	n/a	none	+ M
12-hour PHA Response	none	+ 2011	none	none
Body Condition Score	none	+ 2012	+	none
Fat Body Weight	none	none	+	+ F
Parotid Gland Area	none	- 2011	none	none
Gut Contents	+ (marginal)	none	-	none
Toe Web Scores	none	none	n/a	none

Table 3.3. Summary of full mixed models. Plus and minus signs describe the direction of the significant relationship. For example, a plus sign at the intersection of Body Condition Score and Day reflects that higher body condition scores are associated with capture on later days of the year. Where necessary for categorical variables, the category given matches the direction of the relationship. For instance, "+ 2012" means that the response variable was significantly higher in 2012. "None" describes no significant relationship. "N/A" references variables that were not included in the model.

Response Variable	Pesticide	Year	Day	Sex	PHA	Fat Body	Body Condition
Baseline CORT	none	+ 2011	none	+ M	none	n/a	none
12-hour PHA Response	none	+ 2011	none	none	n/a	n/a	+
Body Condition Score	none	+ 2012	none	none	+	+	n/a
Fat Body Weight	none	none	+	none	none	na	n/a
Parotid Gland Area	none	- 2011	none	none	n/a	n/a	n/a
Gut Contents	+	none	none	none	n/a	n/a	n/a
Toe Web Scores	none	none	n/a	none	none	n/a	n/a

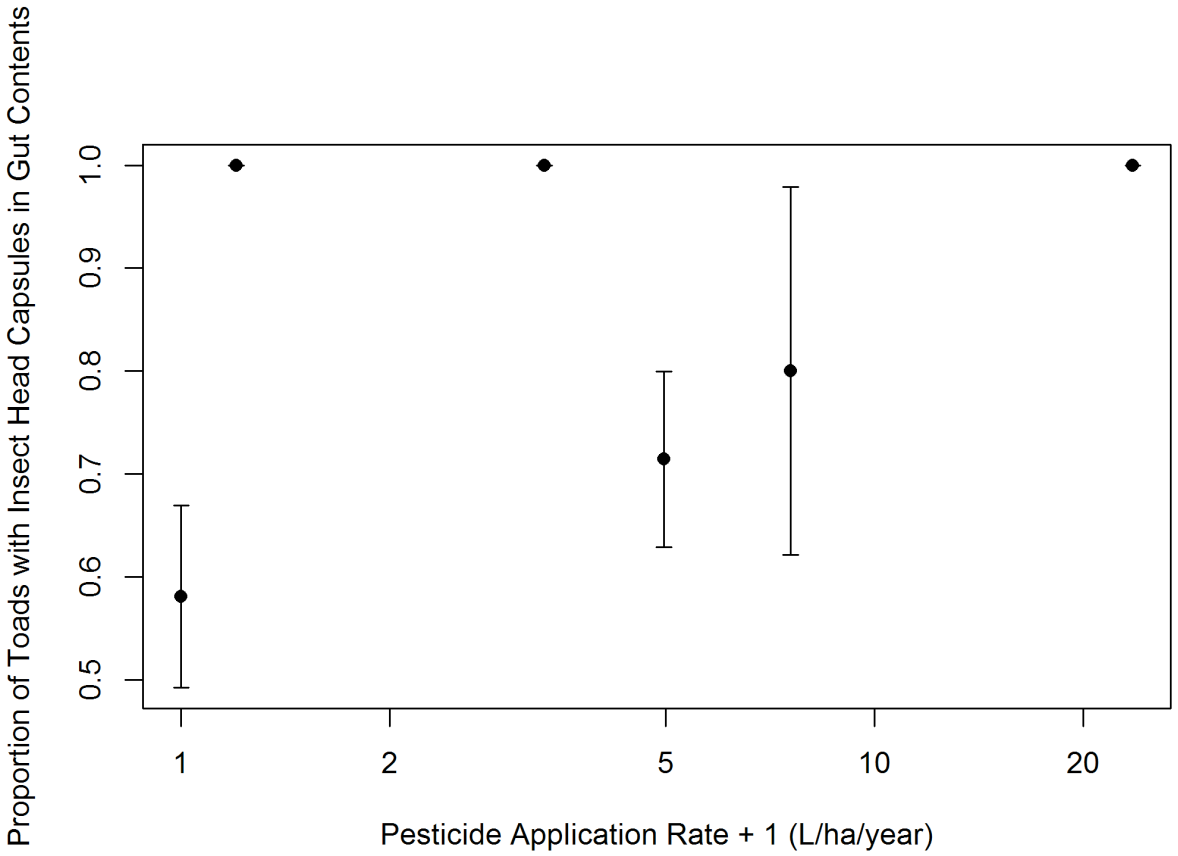

Figure 3.1. Proportion of toads with insect head capsules in gut contents along a gradient of total pesticide application rates. Error bars represent standard error. Error bars could not be calculated for sites where all toads had insect head capsules in gut contents. Sites with higher total pesticide application rates had significantly more toads with insect head capsules in their gut contents (full model p=0.0225).

CHAPTER 4

RELATIONSHIP OF PESTICIDES AND RHINELLA MARINA MACRO- AND MICRO-PARASITE BURDENS[3]

[3]McDowell, K. M. S., S. M. Hernandez, M. J. Yabsley, K. Navara, A. Ellis, and C. R. Carroll. To be submitted to Ecological Applications.

Abstract

Amphibians are declining worldwide, due to a number of factors. Stressors such as disease and pesticide exposure can interact to create non-additive interactive effects. We assessed the micro-parasite prevalence and macro-parasite infection intensity of the cane toad, *Rhinella marina*, in artificial wetlands in Guanacaste, Costa Rica with a variety of pesticide application rates. Our objective was to determine if pesticide exposure was associated with parasite prevalence or infection intensity. Pesticide application rate was a significant predictor of infection intensity for several parasites: *Rhabdias* nematodes, intestinal trematodes, *Hemolivia stellata*, and *Ochoterenella* spp. *H. stellata* burdens were higher in sites with higher pesticide application rates. Burdens for the other three parasites were higher in sites with lower pesticide application rates. Ectoparasites and intestinal nematodes had no significant relationship with pesticide application rate. All individuals tested were negative for Bd and ranavirus. Parasite life history appears to be important in determining how host infection intensities are related to pesticide exposure, and we expect that pesticides may be impacting parasites during life stages outside of the amphibian host. Lower infection intensities of several important parasite species may mitigate any direct stress of pesticide exposure on the toads, and perhaps help to explain their persistence in these habitats.

Introduction

Amphibian populations around the world are declining at alarming rates (Hof et al. 2011; Stuart et al. 2004). The reasons for population declines vary by species and geographic area, but some of the top causes are habitat loss and disease (Collins 2010; Collins and Storfer 2003; Hof et al. 2011). Nowhere has this crisis been felt more than in Central America (Hero and Kriger 2008; Stuart et al. 2004). While the potential causes of amphibian declines have been an active research area in recent years, they remain inherently complex and we are far from understanding all of the variables at play.

At a time when amphibians are already imperiled by infectious diseases, it is important to understand factors that may exacerbate their susceptibility to pathogens or parasites. Micro-parasites alone have caused declines of amphibian populations, and in some cases, even extinctions (Daszak et al. 2003; Gray et al. 2009; Kilpatrick et al. 2010). Additionally, there is evidence that parasites can interact with other stressors in the environment to cause greater effects on the individual hosts (Gallana et al. 2013; Hof et al. 2011; Schotthoefer et al. 2011).

Pesticides have been shown to cause a variety of health effects in amphibians, including gonadal malformations (Hayes et al. 2003; McCoy et al. 2008), altered growth and metamorphosis (Cheek et al. 1999; Glennemeier and Denver 2001; Hayes et al. 2006), and mortality (Mann and Bidwell 1999; Relyea and Diecks 2008; Relyea and Jones 2009). In addition to these direct effects, pesticides can also interact with a variety of other stressors, including parasites (Forson and Storfer 2006; Kerby and Storfer 2009; Kerby et al. 2011). It is therefore important to understand both how pesticides affect amphibians, and to better understand the complex interactions of pesticides and parasites (Lafferty and Kuris 1999).

Pesticides and parasites may interact through the downregulation of a host's immune system (Christin et al. 2003; Christin et al. 2004; Gilbertson et al. 2003; Lafferty and Kuris 1999; Rohr et al. 2008). Using Carey et al.'s (1999) definition of stressor, which is any "deleterious or injurious environmental change to which a species has not evolved the capacity to compensate fully", pesticides can be thought of a "stressor" for amphibians and other non-target organisms. Pesticides may either cause neuroendocrine changes that result in immunosuppression, or directly negatively influence the immune system (Carey et al. 1999). If the negative effects on the immune system are sufficient, pesticides can lead to increased parasite burdens and altered effects of parasite infection, as shown in experimental infection studies following exposure of amphibians to various pesticides (Kiesecker 2002; Rohr et al. 2008; Rohr et al. 2008), as well as in limited field studies (Linzey et al. 2003). Pesticides also have been shown to increase susceptibility to, and subsequent mortality from, at least one amphibian micro-parasite (Forson and Storfer 2006; Kerby and Storfer 2009).

In Costa Rica, where the effect of infectious diseases on amphibians has been more devastating than in most other countries, pesticide usage is extremely high. Costa Rica imports more pesticides per hectare of land than any other country in the world (World Resources Institute 2011). At the same time, natural wetland habitats both in Costa Rica and worldwide are declining, forcing amphibians to seek out alternative habitats to complete their life cycle (Daniels and Cumming 2008; Millenium Ecosystem Assessment 2005; Prigent et al. 2012). In the Guanacaste province of Costa Rica, rice is an important crop, and rice fields are a major landscape feature (Organization for Tropical Studies 2001). Rice fields have been suggested as a viable surrogate habitat for amphibians were natural wetlands are declining (Duré et al. 2008; Machado and Maltchik 2010). However, these agricultural habitats expose amphibians to a

variety of anthropogenic chemicals, including pesticides, which may interact with amphibian parasites to produce unforeseen consequences. This scenario could have particularly troubling repercussions in Costa Rica, which harbors a large proportion of the world's amphibian diversity (174 species, 44 of which are endemic), despite the country's small size of 50,900 km^2 (Savage 2002).

To examine the relationships between pesticide regimes and parasite prevalence and intensity in amphibians in these habitats, we sampled free-living populations of the cane toad, *Rhinella marina* (*Bufo marinus, Chaunus marinus*). The cane toad is native to Central America and northern South America. These large toads are a well-known invasive species throughout much of the world and thrive in human disturbed habitats (Zug and Zug 1979). As habitat generalists with a high degree of adaptability to disturbed habitats, they use artificial wetlands frequently, where they are exposed to a variety of anthropogenic contaminants, including pesticides.

We examined a variety of micro- and macro-parasites of cane toads to develop a comprehensive picture of pesticide-parasite interactions in this system. Micro-parasites included *Batrachochytrium dendrobatidis* (Bd) and ranavirus. Bd, the causative agent of chytridiomycosis, has caused large mortality events of amphibians worldwide (Daszak et al. 1999; Fisher et al. 2009). While die-offs caused by Bd typically involve upland forest amphibians in tropical regions, we assessed Bd infection status because of the global importance of this pathogen and because infections of *R. marina* have been reported (Lips et al. 2006). Ranavirus is another important amphibian pathogen and has caused substantial mortality events in North America, Europe, and Asia (Daszak et al. 2003; Gray et al. 2009), and has recently been reported in Costa Rica (Whitfield et al. 2012; Whitfield et al. 2013).

We also examined several macro-parasites, including lung and intestinal helminths, haemoparasites, and ectoparasites. Lungworms in the genus *Rhabdias* are a common and costly parasite for toads that can reduce survival and growth, reduce prey intake, and impair locomotor performance (Kelehear et al. 2009; Kelehear et al. 2011; Kuzmin et al. 2007). Reduced immune response to *Rhabdias* spp. has also been correlated with agrochemical exposure (Christin et al. 2003). Intestinal helminths, haemoparasites, and ectoparasites are all common parasites of cane toads and their effects on the host vary (Davies and Johnston 2000; Espínola-Novelo and Guillén-Hernández 2008; Esslinger 1988; Esslinger 1989; Goldberg and Bursey 2010; Luz et al. 2013; McKenzie and Starks 2008; Smith et al. 2008). These parasites were measured as they are ubiquitous amphibian parasites that can be considered a part of a healthy ecosystem (Hechinger and Lafferty 2007; Hudson et al. 2006). We also used several host variables to contextualize parasite infections. These included baseline corticosterone, the primary glucocorticoid for herpetofauna (Romero and Wikelski 2001); a phytohemagglutinin (PHA) assay to assess cellular immune function (Martin et al. 2006; Siva-Jothy et al. 2001); and a body condition score calculated from mass and snout-vent length (Bancila et al. 2010; Waye and Mason 2008).

This study aimed to determine the relationship of pesticide exposure with the pathogen and parasite burdens of *R. marina* in artificial wetlands in Guanacaste, Costa Rica. The primary objective was to determine if pesticide exposure was associated with differential prevalence of infection and intensity in a free-living amphibian host. We expected that parasite prevalence and intensity would be higher in sites with higher total pesticide application rates.

Methods

Study Area and Sample Sites

The Rio Tempisque watershed in Guanacaste, Costa Rica is approximately 5,404 sq km extending from the central mountains to the Pacific coast. Land use in the watershed is a mosaic of urban areas, protected areas, and various agricultural crops, with rice being one of the most widespread.

Sample sites consisted of artificial wetlands in the Rio Tempisque watershed near the town of Cañas (Appendix A). We defined artificial wetlands as any area that is flooded due to human activities, whether permanently (e.g., pond) or temporarily (e.g., agriculture). Artificial wetlands used as sample sites included four rice fields, a seasonally flooded woodlot, and an artificial pond and surrounding lawn on the property of a rural ecotourism lodge (Appendix B). Because the beginning of the wet season is the peak breeding period for most local amphibian species, sampling took place during the transition from the dry to the wet season (March to June) in 2010 - 2012.

Sample sites varied in their amount of pesticide application. Pesticide application was estimated using landowner surveys, as explained below. Pesticide exposure varied from none reported to levels typical of commercial rice production. This range of pesticide exposure provided a gradient of exposures in habitats that in all other regards were extremely similar.

Landowner/Manager Surveys

Each landowner or manager answered standardized survey questions regarding the types and quantities of pesticides applied (Appendix C). These data (Appendix D) were then used to calculate the total pesticide application rate per hectare per year for each separately managed site.

Soil Pesticide Concentration Analysis

Soil samples were collected in May - July of 2012. Two samples were collected from each field site, approximately five weeks apart. Samples were a composite collected from the top 25 mm of soil at five locations around the field sites, from areas that were moist but not waterlogged. The soils were placed in 75 mL plastic containers and stored at -20 ^{o} C until export to the UGA Agricultural Services Laboratory, Athens, GA. Samples were received at the UGA Agricultural Services Laboratory, assigned individual identification number and stored at – 4^{o} C until analysis. Soil sample composites were soxhlet extracted with ethyl acetate for 4 hrs. The extract was concentrated on rotary evaporator and made to a final volume of 2 ml. for GLC analysis. The extract was then analyzed for nitrogen and phosphorus-containing pesticides using a Perkin Elmer Autosystem Gas Chromatograph equipped with a NP detector and a ZB-5 Megapore (0.53mm) 30-m column. The column was programmed from 135 to 275^{o} C at 5^{o} C min^{-1}. A fortified sample and reagent blank was included with the sample extraction set. Potential positive residues were quantified by external standardization (3 point calibration) with the lower reporting limit set equal to the lowest calibration standard. The chromatography is a modification of EPA Drinking Water Method 507 adapted to current laboratory analytical systems.

The extract was also analyzed for chlorinated pesticides using a Agilent 7890 series Gas Chromatograph equipped with dual Ni63 electron Capture detectors and a ZB-5 Megapore (0.53mm) 30-m columns. The column was programmed from 135 to 275^{o} C at 5^{o} C min^{-1}. A fortified sample and reagent blank was included with the sample extraction set. Potential positive residues were again quantified by external standardization (3 point calibration) with the lower reporting limit set equal to the lowest calibration standard. The chromatography is a

modification of EPA Drinking Water Method 508 adapted to current laboratory analytical systems.

Amphibian Captures and Sample Collection

All procedures were approved by the University of Georgia's Institutional Animal Care and Use Committee (AUP #A2012 03-011). *R. marina* were captured during 62 sampling dates evenly distributed throughout the sampling period. Toads were held in individual enclosures for 12-36 hours before necropsy, during which time pre-necropsy samples were taken. Toads were humanely euthanized by immersion in solution containing an overdose of buffered tricaine methanesulfonate (MS-222) followed by cervical pithing (American Veterinary Medical Association 2007). Several variables (baseline corticosterone, PHA response, and body condition score) discussed in Chapter 3 were included as additional predictor variables to determine if immune function or body condition are related to parasite prevalence or intensity. Methods for the measurement of these variables are available in Chapter 3.

Parasite Collection and Analysis

Ectoparasites (only ticks were found) were removed from the skin and preserved in 100% ethanol. Blood was collected from either the abdominal midline vein or the heart, and thin blood smears were made, air-dried, fixed with 100% methanol, and stained using a modified Romanowsky stain (Diff Quick®, Jorgensen Laboratories, 1450 Van Buren Ave., Loveland, CO 80538, USA). The entire slide was scanned for microfilaria at 100x, and the monocellular layer was examined at 400x and 1,000x (with oil immersion) for 10 minutes for haemoparasites, which were identified to genus based on morpohology (Desser 2001).

The lungs were fixed in 10% buffered formalin and later dissected to collect *Rhabdias* nematodes. To determine the abundance of intestinal parasites, intestines were linearized, opened

lengthwise, scraped and washed with 100% ethanol into a 100 mesh. Intestinal wash samples were examined under a dissecting microscope and all parasites were identified to phylum and enumerated.

Bd and Ranavirus Sample Collection and PCR Analysis

The ventral surface of each toad was thoroughly swabbed 20 times using a sterile cotton swab to collect DNA for Bd testing following standard sampling techniques (Hyatt et al. 2007). Swabs were frozen at -20˚C dry in sterile microcentrifuge tubes. A 25 mg section of the liver was collected during necropsy and frozen at -20˚C for ranavirus testing. DNA was extracted from swabs and liver tissue using Qiagen DNeasy kits (Qiagen, Venlo, Limburg, Netherlands) following the manufacturer's directions and was tested for Bd or ranavirus by PCR using established methods (Annis et al. 2004; Greer and Collins 2007; Mao et al. 1997).

Histopathology

Tissue sections of all the major organs were collected during necropsy and preserved in 10% buffered formalin. Histopathology slides were examined and scored by a board-certified veterinary pathologist with expertise in herpetofauna. The total number of organs in which detrimental effects due to parasites or pathogens were noted and the number of different parasite taxa identified were counted for each toad. The effects of trematodes detected in the liver were scored by the following system: 0 = no significant lesion observed; 1 = trematodes and/or bile duct dilation only (no inflammation, hyperplasia, or fibrosis); 2 = biliary epithelial hyperplasia, branching, or inflammation; 3 = peribiliary fibrosis.

Statistical Analysis

Quantitative soil pesticide concentrations in soil samples were below detection limits; therefore, data were analyzed using total pesticide application rates. Pesticides could not be

analyzed individually or by group, as these measures covaried with total application rates. Total pesticide application rates were calculated as total liters of herbicides and insecticides applied per hectare per year. The log of total pesticide application rates was used, due to a right skewed distribution. Data were analyzed using general linear mixed-effects models with site as a random effect. Fixed effects varied depending on the response variable, but total pesticide application rate, year captured, day of year captured, and sex of individual were always included. The only exception to this procedure was that year captured was not included for *H. stellata* or *Ochoterenella*, because burdens for these parasites were only assessed for toads captured in 2012. Two sets of models were run for each response variable, one including only the fixed effects mentioned above (referred to as basic model), and one including other effects that could have biological relationships with the response variable (referred to as full model). Additional predictive variables included other measured variables discussed in this paper, as well as additional variables discussed in Chapter 3. The number of samples per predictive variable was always $\geq$10, to avoid overparameterization of the models (Vittinghoff and McCulloch 2007). A Pearson Correlation matrix was completed to ensure that auto-correlated variables were not included as effects in the same model. For each set of models, a stepwise regression was employed to eliminate the variable with the highest p-value until the remaining model contained only significant effects (p-value$\leq$0.05). Bd and ranavirus infection status were not analyzed, since all individuals tested were negative for both pathogens. Generally, linear regressions were used, but an ordinal logistic regression was used for the liver lesions histopathology analysis. All analyses were completed using the program R version 2.14.1 with package lme4, except for the liver lesions histopathology analysis, which was completed using package ordinal.

Results

A total of 100 *R. marina* were captured. The number of toads varied for which each response variable was measured (Table 4.1), thus the sample size in the full models sometimes became larger during the stepwise regression, as predictor variables with smaller sample sizes than the response variable were removed. Predictor variables with much smaller sample sizes than the response variable were excluded whenever possible; however, variables expected to have a strong biological relationship were included in the full model regardless of sample size. The results of both the basic and full models are reported here to avoid losing the power of the full sample size collected (Tables 4.2, 4.3). In cases where the results of the basic and full models differ, this effect is due to the process of the stepwise regression. Using both sets of models allows us to examine the importance of a variety of predictor variables without losing the emphasis on our predictor variables of primary interest.

Ectoparasites

In the basic model for ectoparasites (n=84), year captured (p-value=0.001169), day of year captured (p-value=0.000831), and sex (p-value<0.00001) were all significant predictors. Year captured and day of year captured were the only significant predictors in the full model (p-values=0.033228, 0.000479, respectively, n=84). Toads captured in 2012 had significantly fewer ticks than toads in 2011, and toads captured on earlier days of the year had more ticks than those captured on later days. Male toads had significantly more ticks than females.

Rhabdias *spp. Lung Nematodes*

Year captured (p-value<0.00001), day of year captured (p-value<0.00001), sex (p-value<0.00001), and total pesticide application rate (p-value=0.0356) were all significant predictors of *Rhabdias* lung nematode burdens in the basic model (n=100) (Figure 4.1). Higher

pesticide application rates and males were associated with lower *Rhabdias* burdens. Toads captured in 2012 had significantly higher *Rhabdias* burdens than toads captured in 2010 or 2011, and toads captured on earlier days of the year had higher *Rhabdias* burdens than those captured later in the year. In the full model, year captured (p-value<0.00001), sex (p-value<0.00001), corticosterone concentration (p-value<0.00001), and 12-hour PHA inflammatory response (p-value=0.0035) were all significant predictors (n=48). Toads captured in 2012 had significantly higher *Rhabdias* burdens than toads captured in 2011. Toads with higher baseline CORT and lower PHA inflammatory response had higher *Rhabdias* burdens. Female toads had significantly higher *Rhabdias* burdens than males.

Intestinal Nematodes

Year captured (p-value=0.00357), day of year captured (p-value=0.04943), and sex (p-value=0.01171) were all significant predictors of intestinal nematode burdens in the basic model (n=84). The full model results showed year captured (p-value<0.00001), corticosterone concentration (p-value=0.00354), and 12-hour PHA inflammatory response (p-value=0.04407) as significant predictors (n=48). Toads captured in 2012 had higher intestinal nematode burdens than toads captured in 2011. Toads captured on earlier days of the year and females had higher intestinal nematode burdens than toads captured on later days and males. Higher baseline CORT and higher 12-hour PHA inflammatory response were associated with higher intestinal nematode burdens.

Intestinal Trematodes

In the basic model for intestinal trematode burdens, day of year captured (p-value<0.00001), sex (p-value=0.003327), and total pesticide application rate (p-value=0.000134) were significant predictors (n=84) (Figure 4.2). High trematode burdens were associated with

habitats with lower pesticide application rates and male toads. Toads captured on later days of the year had significantly higher intestinal trematode burdens than toads captured on earlier days. In the full model, only 12-hour PHA inflammatory response was a significant predictor (p-value=0.00666, n=66). Higher 12-hour PHA inflammatory response was associated with higher intestinal trematode burdens.

Hemolivia stellata

Day of year captured and total pesticide application rates were significant predictors of *H. stellata* burdens in the basic model (p-values=0.000304, 0.049777, respectively, n=38) (Figure 4.3). Higher pesticide application rates and captures on earlier days of the year were associated with higher *H. stellata* burdens. In the full model, body condition score and 12-hour PHA inflammatory response were the only significant predictors (p-values=0.000802, 0.001235, respectively, n=21). Toads with lower body condition scores and lower 12-hour PHA inflammatory response had higher *H. stellata* burdens.

Ochoterenella *spp.*

In the basic model, day of year captured (p-value<0.00001), sex (p-value<0.00001), and total pesticide application rate (p-value=0.0154) were all significant predictors of *Ochoterenella* burdens (n=38) (Figure 4.4). Sex (p-value=0.03382), total pesticide application rate (p-value=0.03585), and body condition score (p-value<0.00001) were significant predictors in the full model (n=38). In both models, high *Ochoterenella* burdens were associated with habitats with lower pesticide application rates. Toads captured on later days of the year had significantly higher *Ochoterenella* burdens than toads captured on earlier days. Higher body condition scores were associated with higher *Ochoterenella* burdens. In the basic model, female toads were associated with higher *Ochoterenella* burdens, however in the full model, male toads were

associated with higher burdens. This inconsistency is most likely due to the stepwise approach, and the changing sample size in the full model. For this reason, we believe that the results from the basic model are more dependable.

Histopathology

Day of year captured was a significant predictor of the number of organs affected by parasites in both the basic and the full models (p-value=0.0327, n=100). Toads captured on later days of the year had significantly fewer organs affected. Day of year captured was also a significant predictor of liver lesion scores in both the basic and the full models (p-value<0.00001, n=99). Toads captured on later days of the year had significantly lower liver lesion scores. For the number of parasite taxa identified, no variable was a significant predictor (n=100).

Bd and Ranavirus

Neither Bd nor ranavirus were detected in any of the toads sampled (n=100, 84, respectively).

Discussion

We found that pesticide exposure was related to the intensity of parasites in *R. marina*. Higher total pesticide application rates were associated with higher *H. stellata*, yet lower *Rhabdias* spp., intestinal trematode, and *Ochoterenella* spp. burdens. However, for ticks and intestinal nematodes, total pesticide application rate was not a significant predictor of infection burdens.

Parasite loads can increase in stressed amphibian populations, including those exposed to high concentrations of environmental toxicants and agrochemicals (Daszak et al. 1999; Kelehear et al. 2009). For example, pesticides interact with *Ambystoma tigrinum* virus, a ranaviral

pathogen, leading to increased susceptibility and mortality (Forson and Storfer 2006; Kerby and Storfer 2009; Kerby et al. 2011). In addition, pesticide exposure can increase trematode infections in both laboratory and field studies (Rohr et al. 2008; Rohr et al. 2008). These studies indicate that systems with high agrochemical exposure might be expected to harbor amphibian hosts with high parasite burdens.

In contrast, parasites can be lost from an "unhealthy" ecosystem, such as systems with high pesticide exposure, due to impacts on definitive hosts, intermediate hosts and/or vectors. This is especially true for parasites that have complex life cycles which are highly dependent on high biodiversity (Hechinger and Lafferty 2007). In fact, it has been suggested that parasites can be used as an indication of polluted environments where few parasites are to be expected (Hatcher et al. 2012) or to evaluate restoration projects, which should harbor high numbers of parasites within a healthy ecosystem (Huspeni and Lafferty 2004). However, because anthropogenic impacts, such as pesticide exposure, can affect both hosts and parasites, the ultimate impacts on parasite prevalence and infection intensity will be dependent on which is affected more, the host or the parasite (Lafferty 1997; Lafferty and Holt 2003; Lafferty and Kuris 1999). Because outcomes are highly context dependent, it is logical that not all parasites will share the same outcome.

Among the parasites examined, we found that infection intensities of parasites with complex life cycles were lower in sites with high pesticide application rates. *H. stellata* are the exception to this, and it is possible that either they or their vectors are more resistant to pesticide exposure. For instance, the parasites sampled in this study have a variety of life history strategies. *Rhabdias* spp. nematodes, which parasitize the lungs, have a direct life cycle and free-living life stages (Kelehear et al. 2009; Kelehear et al. 2011). Intestinal trematodes have an

indirect life cycle and depend on intermediate hosts, usually snails (Espínola-Novelo and Guillén-Hernández 2008; Goldberg and Bursey 2010). Intestinal nematodes have varied life histories, but generally have direct life cycles and infect amphibians either by being ingested or through direct penetration of the skin (Goldberg and Bursey 2010). Although ticks must attach to a host to obtain a blood meal, they spend a large part of their life cycle in the environment in between blood meals (Luz et al. 2013). Haemogregarines are vector-borne protozoa, and are typically transmitted by leeches (Davies and Johnston 2000), although the vectors for *H. stellata* are ticks (Lainson et al. 2007). Microfilaria are vector-borne filarial worm larvae, and are transmitted by flies and mosquitoes (Desser 2001; McKenzie and Starks 2008), although the vector for *Ochoterenella* spp. in Central America is unknown.

Year captured was a significant predictor for ticks, *Rhabdias*, and intestinal nematodes. Tick burdens were higher on toads caught in 2011, while *Rhabdias* and intestinal nematode burdens were higher in toads caught in 2012. Year as a significant predictor reflects stochastic interannual variation in habitat, such as precipitation. Day of year captured was a significant predictor for every parasite analyzed. Toads captured on later days of the year had higher *Ochoterenella* and intestinal trematode burdens, but lower intestinal nematode, *Rhabdias*, tick and *H. stellata* burdens. This relationship implies that seasonality is important for these parasites, but that different taxa have different patterns of seasonality. Unfortunately, climate data is not publicly available in Costa Rica for us to analyze this relationship. Early sampling days took place during the end of the dry season, while later sampling days were during the beginning of the wet season. Day of year captured was also a significant predictor of the number of organs affected by parasites histopathologically and liver lesion scores. There were no significant predictors of the number of parasite taxa identified by histopathology.

Sex was a significant predictor for every parasite except *H. stellata*. Intestinal trematode and tick burdens were higher in male toads, while intestinal nematode, *Rhabdias*, and *Ochoterenella* burdens were higher in female toads. For *Ochoterenella*, the sex with higher burdens differed between the full and basic models. Sex differences may reflect parasite preference or differences in physiological vulnerability or exposure rate, since there is no particular pattern to which sex is more infected overall. In another study, we found no sex difference in 12-hour PHA inflammatory response used as a measure of cell-mediated immune function, although male toads had significantly higher baseline corticosterone (Chapter 3). Therefore, it is unlikely that a difference in immune function between the sexes is responsible for the differences in infection intensities.

All individuals tested were negative for Bd and ranavirus. While Bd is incredibly important for many amphibian communities in Costa Rica (Lips 1998; Lips et al. 2008), it is generally found in the cool moist highlands. Our study sites are in hot dry lowland forest, and as such are most likely outside of the environmental range for the pathogen (Fisher et al. 2009; Kilpatrick et al. 2010; Murray et al. 2011). Ranavirus has only recently been reported in Costa Rica (Whitfield et al. 2012; Whitfield et al. 2013), and only at La Selva Biological Station in a lowland wet forest. While it is likely that it is only a matter of time before ranavirus is identified in other locations in Costa Rica, it does not appear to be present in our study sites.

Higher *Rhabdias* burdens were associated with higher baseline corticosterone and lower 12 hour PHA inflammatory response. This relationship could mean that *Rhabdias* are better able to infect toads that are stressed and have low cell-mediated immune function, or that the nematodes themselves are stressful for the toads. *Rhabdias* spp. nematodes have been shown to have a high fitness cost for cane toads. Kelehear et al. have demonstrated that *Rhabdias* infection

can reduce survival and growth rates, impaired locomotor performance, and reduced prey intake for metamorph cane toads (2009), and reduce growth rates of adult toads (2011). This reduction in locomotion could also result in toads staying in areas with high *Rhabdias* burdens, leading to a positive feedback of increased intensities. Graham et al. (2012) investigated the effect of captive stress on response corticosterone levels and immune function of *R. marina*, and assessed *Rhabdias* infection status as a covariate. They discovered that response corticosterone was lower in toads infected with *Rhabdias* than in those with no infection. As baseline and response corticosterone are generally expected to show opposite patterns in stressed organisms (Busch and Hayward 2009), this supports our findings that higher *Rhabdias* infection intensities are associated with higher baseline corticosterone. It is likely that these nematodes have a high cost for infected toads, and that changes in corticosterone levels are a result.

Higher intestinal nematode and intestinal trematode burdens were associated with a higher 12-hour PHA inflammatory response. This positive correlation may seem contradictory, as higher PHA inflammatory response is ostensibly related to a higher immune response. However, the PHA test is primarily associated with the cellular arm of the immune system (Brown et al. 2011; Martin et al. 2006). Macroparasites, such as nematodes and trematodes, elicit a humoral immune response in the host (Lee 2006). Because these two components of immunity tradeoff due to energy limitations (Lee 2006), toads with a high cellular immune response may not be able to mount a high humoral response, leading to high macroparasite burdens. Higher intestinal nematode burdens were also associated with higher baseline corticosterone. This relationship could mean that intestinal nematodes are better able to infect stressed toads. High baseline corticosterone may be affecting the ability to mount a humoral response. Chronic

elevated baseline corticosterone can suppress the immune function, leading to increased parasite burdens (Busch and Hayward 2009).

Higher *H. stellata* burdens were associated with lower body condition scores and lower 12-hour PHA inflammatory response. This relationship could mean that *H. stellata* are better able to infect toads in poor condition and with low cell-mediated immunity. This idea is reasonable, since a protozoan parasite such as *H. stellata* would elicit a cell-mediated immune response (Lee 2006). Higher *Ochoterenella* burdens were associated with higher body condition scores. It may be that toads infected with *Ochoterenella* that are in poor condition are not able to survive the infection, and are therefore not present in the environment to be sampled.

One limitation of our study was that we were unable to quantify pesticide concentrations at our study sites. The pesticide residues in our soil samples were below the detection limits, and we were therefore unable to use quantitative concentrations for our analyses. We did not attempt to time the collection of our soil samples with pesticide application in the fields, which may have limited our ability to detect pesticide residues in the soil. Although tissue sample were taken during necropsy, tissue was not analyzed for pesticide concentrations. Subcutaneous fat bodies are the most appropriate tissue for some pesticide testing, and many toads had very small or nonexistent fat bodies. Because the distribution of toads with no fat bodies was uneven among our sample sites, it was impossible to perform an unbiased analysis of pesticide concentrations in toad fat bodies. Fat body analyses are discussed further in Chapter 3. It was also impossible to analyze whole body pesticide concentrations, as substantial tissue samples were required for parasite analyses.

Overall, of the relationships that were significant, most parasites were present in lower intensities in habitats with higher pesticide exposure, including the parasite most likely to cause

significant harm to the host, *Rhabdias*. This result suggests that *R. marina* may experience some degree of parasite release in high pesticide environments. However, it is unknown whether this advantage is outweighed by other potentially detrimental effects of pesticides. Pesticides cause effects not only on parasite burdens, but also on growth, time to metamorphosis, and overall survival in amphibians (Forson and Storfer 2006; Glennemeier and Denver 2001; Hayes et al. 2006; Kerby and Storfer 2009; Kerby et al. 2011; Mann and Bidwell 1999; Relyea and Jones 2009). These effects are particularly important for neotropical amphibian species, which have some of the fastest rates of decline in the world, and have been disproportionately affected by disease (Stuart et al. 2004). Further studies should determine whether these effects take place in natural systems, and their net effect on amphibian populations.

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Table 4.1. Summary statistics for response variables.

Response Variable	Average	Standard Deviation	Minimum	Maximum	Number of Toads Measured
Ectoparasites	5.833	23.494	0	212	84
Rhabdias Lung Nematodes	8.892	12.917	0	67	100
Intestinal Nematodes	2.786	6.108	0	36	84
Intestinal Trematodes	0.643	3.963	0	36	84
Haemogregarines	1.895	3.220	0	9	38
Microfilaria	3.763	8.188	0	31	38
Affected Number of Organs	2.890	1.043	0	6	100
Parasite Taxa Identified	1.340	0.956	0	4	100

Table 4.2. Summary of basic mixed models. Plus and minus signs describe the direction of the significant relationship. For example, a plus sign at the intersection of *Rhabdias* and CORT reflects that higher *Rhabdias* burdens are associated with higher CORT values. Where necessary for categorical variables, the category given matches the direction of the relationship. For instance, "+ 2012" means that the response variable was significantly higher in 2012. "None" describes no significant relationship. "N/A" references variables that were not included in the model.

Response Variable	Pesticide	Year	Day	Sex
Ectoparasites	none	+ 2011	-	+ M
Rhabdias spp.	-	+ 2012	-	+ F
Intestinal Nematodes	none	+ 2012	-	+ F
Intestinal Trematodes	-	none	+	+ M
Hemolivia stellata	+	n/a	-	none
Ochoterenella spp.	-	n/a	+	+ F
Affected Number of Organs	none	none	-	none
Parasite Taxa Identified	none	none	none	none
Liver Lesion Scores	none	none	-	none

Table 4.3. Summary of full mixed models. Plus and minus signs describe the direction of the significant relationship. For example, a plus sign at the intersection of *Rhabdias* and CORT reflects that higher *Rhabdias* burdens are associated with higher CORT values. Where necessary for categorical variables, the category given matches the direction of the relationship. For instance, "+ 2012" means that the response variable was significantly higher in 2012. "None" describes no significant relationship. "N/A" references variables that were not included in the model.

Response Variable	Pesticide	Year	Day	Sex	CORT	Body Condition	PHA
Ectoparasites	none	+ 2011	-	none	none	none	none
Rhabdias spp.	none	+ 2012	none	+ F	+	none	-
Intestinal Nematodes	none	+ 2012	none	none	+	none	+
Intestinal Trematodes	none	none	none	none	none	none	+
Hemolivia stellata	none	none	none	none	n/a	-	-
Ochoterenella spp.	-	none	none	+ M	n/a	+	none
Affected Number of Organs	none	none	-	none	none	none	none
Parasite Taxa Identified	none	none	none	none	none	none	none
Liver Lesion Scores	none	none	-	none	none	none	n/a

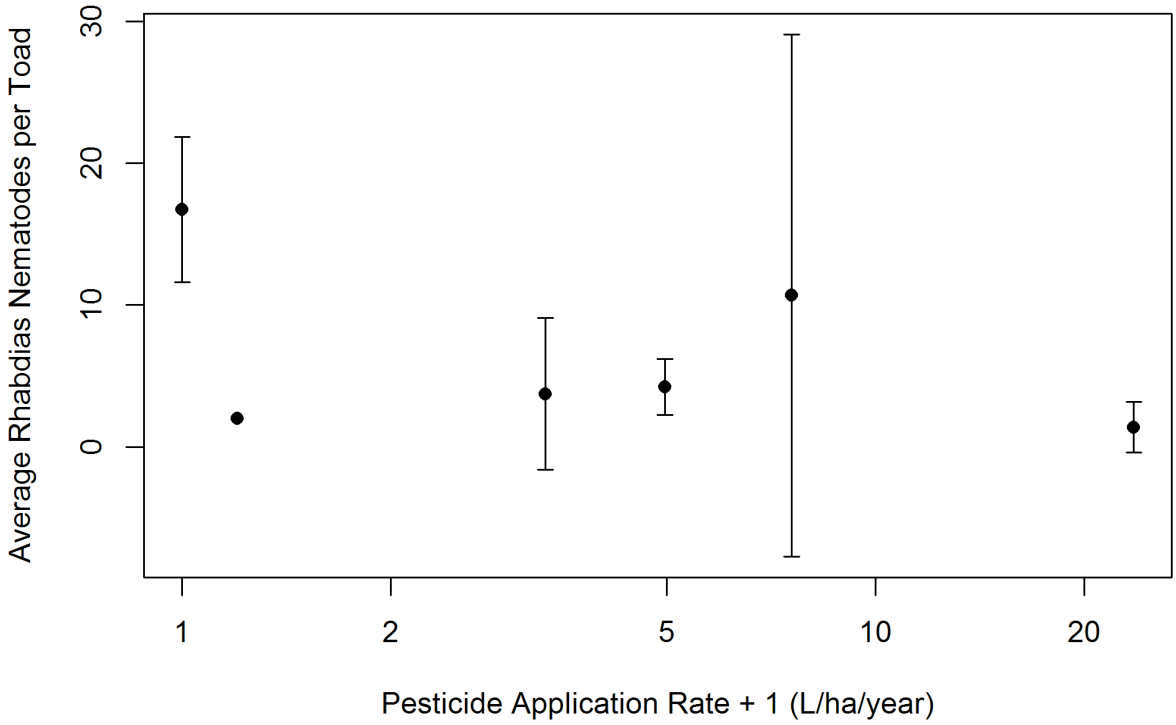

Figure 4.1. Average *Rhabdias* spp. nematode burden per toad along a gradient of total pesticide application rates. Error bars represent 95% confidence intervals. Sites with higher total pesticide application rates had significantly lower *Rhabdias* spp. nematode burdens (basic model p=0.0356).

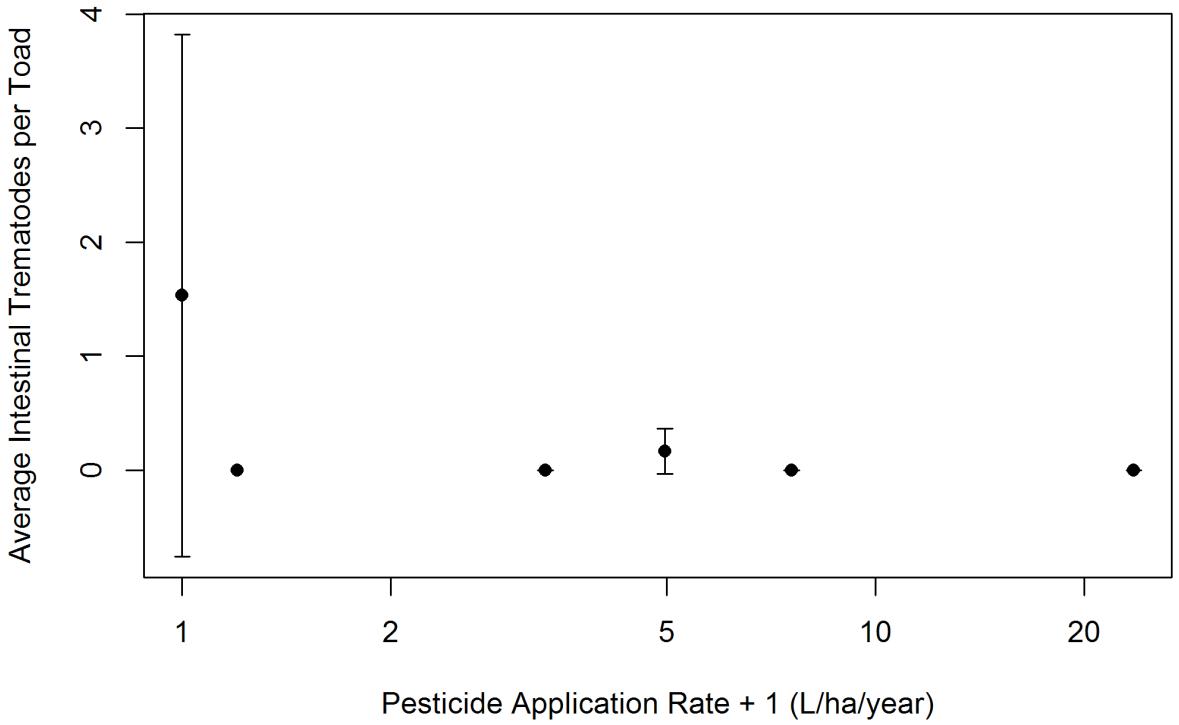

Figure 4.2. Average intestinal trematode burden per toad along a gradient of total pesticide application rates. Error bars represent 95% confidence intervals. Sites with higher total pesticide application rates had significantly lower intestinal trematode burdens (basic model p=0.000134).

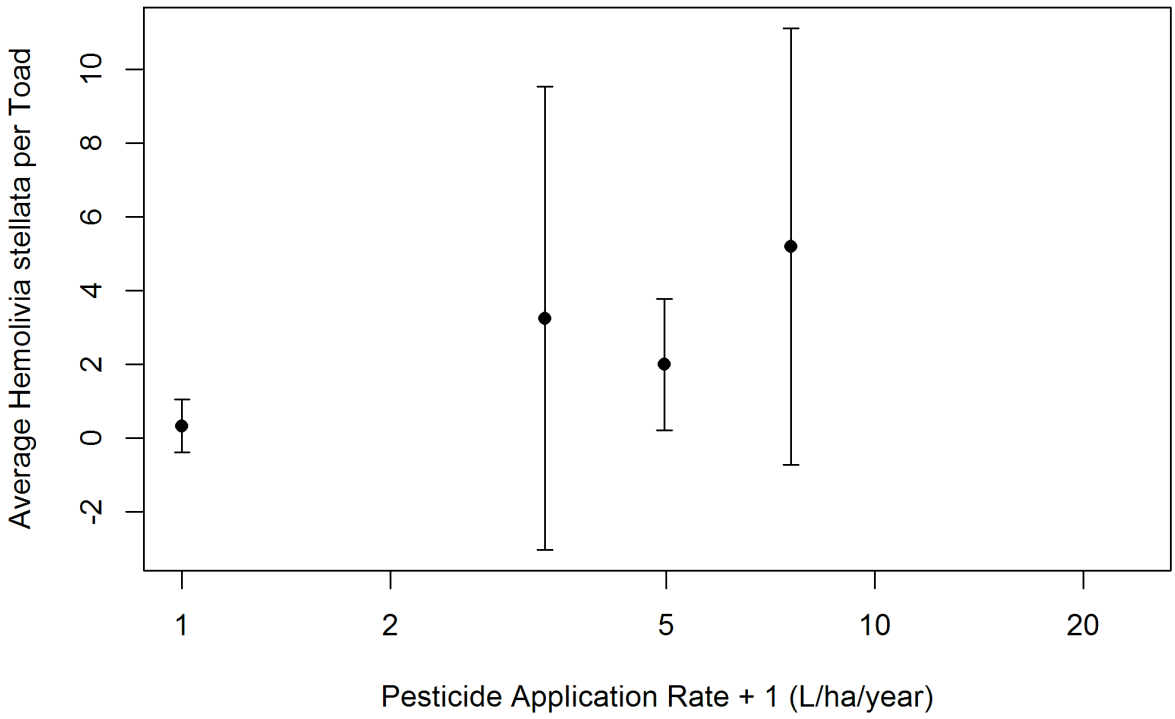

Figure 4.3. Average *Hemolivia stellata* burden per toad along a gradient of total pesticide application rates. Error bars represent 95% confidence intervals. Sites with higher total pesticide application rates had significantly higher *Hemolivia stellata* burdens (basic model p=0.049777).

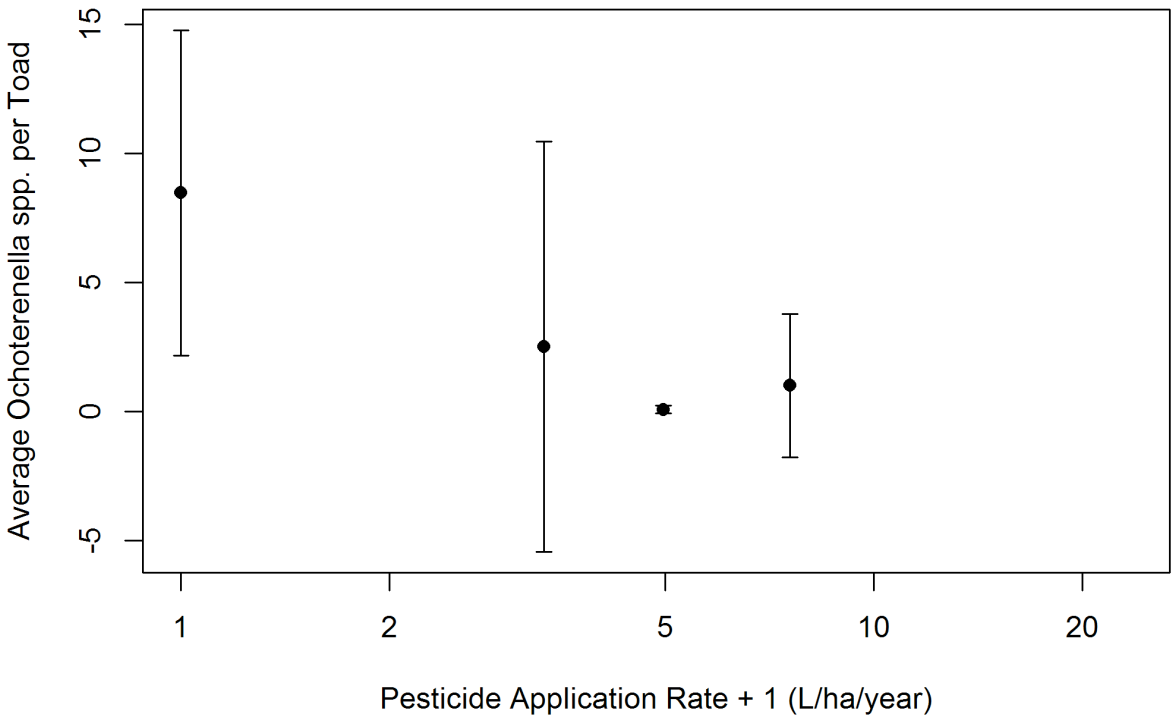

Figure 4.4. Average *Ochoterenella* spp. burden per toad along a gradient of total pesticide application rates. Error bars represent 95% confidence intervals. Sites with higher total pesticide application rates had significantly lower *Ochoterenella* spp. burdens (basic model p=0.0154).

CHAPTER 5

CONCLUSIONS

For the general amphibian community, we found that community composition was similar among sites, but sites with high pesticide application rates generally contained communities that were less similar through time. Higher population densities were associated with lower pesticide application rates for two species, *Leptodactylus melanonotus* and *Rana vaillanti*. Pesticide exposure was significantly related to body condition for one of the six species assessed, *Leptodactylus fragilis*. Higher body condition for this species was associated with lower pesticide application rates. All individuals tested were negative for Bd.

The in-depth study of *Rhinella marina* showed that pesticide application rate significantly predicted the presence of insect head capsules in toad gut contents. Pesticide application rate was also significantly associated with infection intensity for several parasites: *Rhabdias* spp. nematodes, intestinal trematodes, *Hemolivia stellata*, and *Ochoterenella* spp. Higher *Hemolivia stellata* burdens were associated with higher pesticide application rates, while burdens for the other three parasites showed the opposite trend. Several other variables, including those for immune function and body condition, showed no relationship with pesticide application rates. All individuals tested were negative for Bd and ranavirus.

These results suggest that while pesticide exposure may be an important habitat variable for amphibians, importance varies among species. These differences are most likely due to differences in life history leading to variable exposure or susceptibility. Our results, using both a single amphibian species as a model organism, and a wider community study, highlight the

difficulty of extrapolating to the community level from a study of one species. The majority of toxicology studies on amphibians have been conducted on North American anuran species (Hayes et al. 2003; Relyea 2005; Rohr and Crumrine 2005). The wide range of life history strategies and the worldwide distribution of amphibians (Frost et al. 2006; Stuart et al. 2004) may necessitate a wider sampling effort to truly understand how amphibians are affected by these contaminants.

The complexity of natural systems makes predicting the effects of pesticides on natural assemblages difficult. While laboratory and mesocosm studies have detailed a variety of effects of pesticide exposure on amphibians, these effects are not always observed in field studies. In fact, hierarchical studies have shown that even mortality observed in the laboratory and mesocosms can disappear under more natural conditions (Chen et al. 2004; Edginton et al. 2004; Thompson et al. 2004; Wojtaszek et al. 2004). As more field studies are conducted on naturally occurring assemblages of amphibians, the refinement and standardization of methods and measured endpoints should allow researchers to determine with more certainty how amphibian populations will be affected in the long-term by agrochemical exposure.

Future research should focus on determining whether the effects of pesticide exposure on amphibians lead to population declines or changes in community composition or biodiversity in natural systems. The multiple threats facing amphibians and the resulting rapid declines around the world (Stuart et al. 2004) necessitate continued progress on these fronts to inform conservation efforts. Changes in the management of artificial wetlands could make these habitats more suitable to the long-term persistence of amphibian communities, providing an alternative for natural wetlands where this habitat is declining. Studies on relevant habitat features such as hydroperiod, habitat size, and agrochemical use would facilitate the construction of realistic

management options (Humbert et al. 2007; McIntyre et al. 2011; Paton and Crouch 2002). The continued spread of human-altered ecosystems and anthropogenic impacts (Foley et al. 2007; Ramankutty et al. 2008; Vitousek et al. 1997) will require amphibians to survive in these impacted habitats if they are to survive anywhere.

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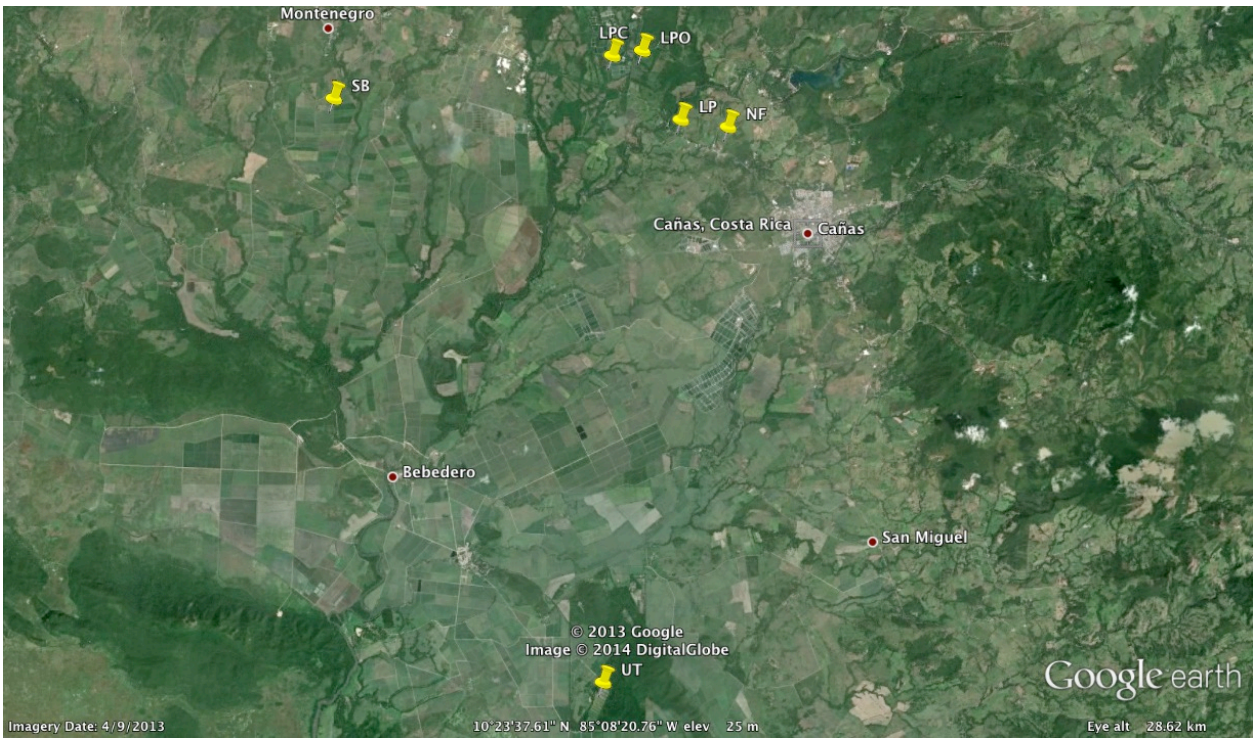
APPENDIX A: MAP OF COSTA RICA WITH CAÑAS MARKED

APPENDIX B: MAP OF STUDY SITES



APPENDIX C: LANDOWNER/MANAGER SURVEY

Date:
Field Site:

1. What herbicides are used in the fields and number of liters used per year?

2. What insecticides are used in the fields and number of liters used per year?

3. What is the total size of the property in hectares?

APPENDIX D: PESTICIDE INFORMATION

Table 1. Summary of names and application rates of pesticides used in each field site, divided into pesticide types. Note: Names of pesticides provided here are those given by the landowners/managers surveyed. In some cases, names provided are trade names, while others are chemical names, which may result in overlap among chemicals. In particular, the active ingredient of Nominee is bispyribac-sodium, the active ingredients of Muralla are imidacloprid and a pyrethroid, and the active ingredient of Clincher is cyhalofop.

	Pesticide Type and Application Rate (L/ha/yr)		
Site	**Herbicides**	**Insecticides**	**Fungicides**
LP	n/a	n/a	n/a
LPO	n/a	Piretroide (0.2)	n/a
NF	Tordon (1.8)	Parasitol (0.18)	n/a
	Glyphosate (0.36)		
LPC	Bispyribac-sodium (0.12)	Piretroide (0.2)	n/a
	Glyphosate (3.5)	Muralla (0.25)	
	Pendimethalin (2.5)		
UT	Clomazone (0.33)	Cipermethrin (0.67)	n/a
	Pyrazosulfuron-ethyl (0.17)	Imidacloprid + cyfluthrin (0.33)	
	Metsulfuron-methyl (1.67)		
	Cyhalofop (0.67)		
	Bispyribac-sodium (0.13)		
SB	Clincher (1)	Tigre (0.6)	Silvacur (0.7)
	Nominee (0.3)	Muralla (1)	
	Garlon (1)	Endosulfan (2)	
	Basagran (4)	Cipermethrin (1)	
	Glyphosate (9)		
	Tordon (2)		

Table 2. List of pesticides and summary of relevant information for each. Note: Names of pesticides provided here are those given by the landowners/managers surveyed. In some cases, names provided are trade names, while others are chemical names, which may result in overlap among chemicals. In particular, the active ingredient of Nominee is bispyribac-sodium, the active ingredients of Muralla are imidacloprid and a pyrethroid, and the active ingredient of Clincher is cyhalofop.

Pesticide	Pesticide Type	Chemical Class	Number of Sites Where Used	Range of Application Rates (L/ha/yr)
Basagran	Herbicide	Benzothiadiazole	1	4
Bispyribac-sodium	Herbicide	Pyrimidinyloxybenzoic	2	0.12-0.13
Clincher	Herbicide	Aryloxyphenoxy propionic acid	1	1
Clomazone	Herbicide	Isoxazolidinone	1	0.33
Cyhalofop	Herbicide	Aryloxyphenoxy propionic acid	1	0.67
Garlon	Herbicide	Chloropyridinyl	1	1
Glyphosate	Herbicide	Phosphanoglycine	3	0.36-9
Metsulfuron-methyl	Herbicide	Sulfonylurea	1	1.67
Nominee	Herbicide	Pyrimidinyloxybenzoic	1	0.3
Pendimethalin	Herbicide	Dinitroanaline	1	2.5
Pyrazosulfuron-ethyl	Herbicide	Sulfonylurea	1	0.17
Tordon	Herbicide	Pyridinecarboxylic	2	1.8-2
Cipermethrin	Insecticide	Pyrethroid	2	0.67-1
Endosulfan	Insecticide	Organochlorine	1	2
Imidacloprid + cyfluthrin	Insecticide	Neonicotinoid and pyrethroid	1	0.33
Muralla	Insecticide	Neonicotinoid and pyrethroid	2	0.25-1
Parasitol	Insecticide	Organophosphate	1	0.18
Piretroide	Insecticide	Pyrethroid	2	0.2
Tigre	Insecticide	Organophosphate	1	0.6
Silvacur	Fungicide	Triazole	1	0.7

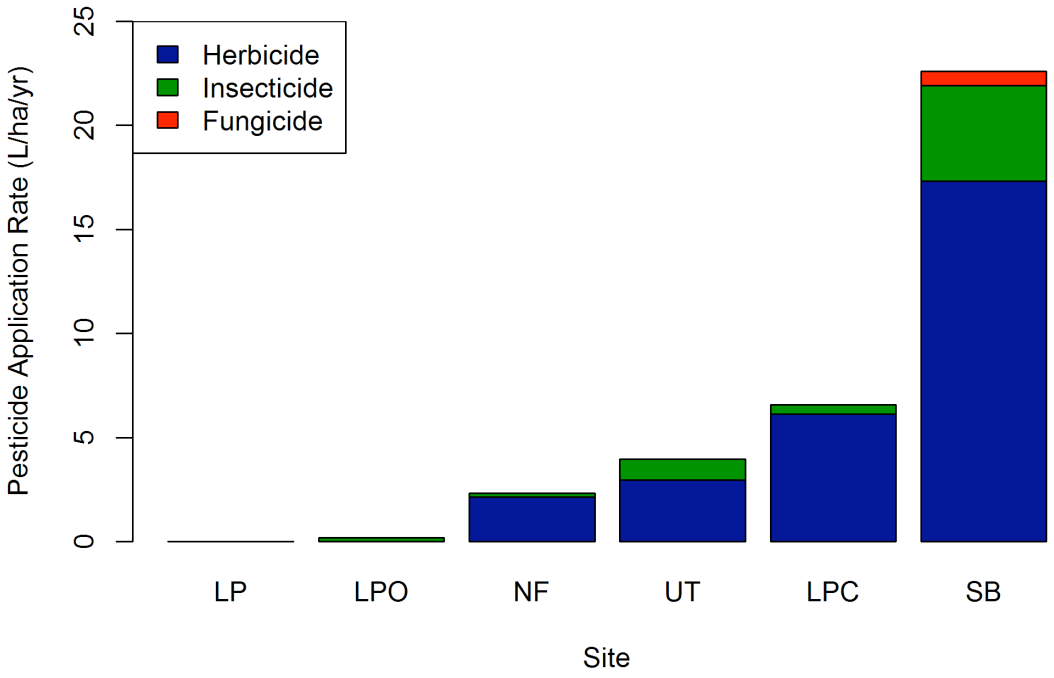


Figure 1. Pesticide application rates by site, divided into pesticide types.