ASSESSING THE IMPACTS OF AGRICULTURAL LAND USE ON WOOD FROG (*LITHOBATES SYLVATICUS*) PRESENCE AND HEALTH IN CENTRAL SASKATCHEWAN

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ABSTRACT

Globally, amphibian populations are declining in response to many factors including habitat loss and degradation, environmental contamination, invasive species, emerging diseases, climate change, and overexploitation. Amphibians are particularly susceptible to habitat loss and contaminants because of their diverse habitat requirements and unique life histories and ecologies. The Canadian Prairie Pothole Region (PPR) is home to several amphibian species, but they are threatened by large-scale conversion of habitat to agriculture. One of the more common amphibians in this region is the wood frog (*Lithobates sylvaticus*), a wide-ranging species that occupies a variety of ecosystems, from forests to prairies to tundra. This makes it an ideal model species to compare results in ecological and toxicological studies. Given anecdotal reports of their abundance in the PPR and simultaneous exposure to a number of anthropogenic stressors, I investigated the effects of environmental variables across multiple scales on wood frog presence and tadpole and metamorph health in central Saskatchewan.

I visited wetlands at five sites near Saskatoon, SK along a gradient of agricultural intensity with two grassland sites (Allan and St. Denis) and three cropland sites (Burr, Colonsay, and Humboldt). I collected data on water quality including nutrients and pesticides, wetland habitat, and surrounding land use and used environmental DNA (eDNA) to detect the presence of ranavirus and wood frogs. To assess the effects of these variables on both wood frog presence and health (condition, mass, and neutrophil to lymphocyte (N:L) ratios), I used boosted regression trees, a relatively novel but growing modelling technique in the ecological sciences.

Wood frogs were present in both grassland and cropland sites. eDNA was more successful at detecting wood frogs in wetlands compared to traditional survey methods – visual encounter surveys and dipnetting. However, for both wood frogs and ranavirus, detection varied seasonally with greater success in the summer than in the spring. Several environmental variables influenced wood frog presence, the most influential being those associated with wetland productivity, vegetation buffer width, and proportion of the surrounding landscape that is comprised of other waterbodies. Wood frog presence was positively associated with higher dissolved phosphorus ($\geq$ 0.4 mg/L), a range of dissolved nitrogen (0.1 to 0.2 mg/L), lower chlorophyll a ($\leq$ 15 µg/L), wider vegetation buffers ($\geq$ 10 m), and more water on the landscape ($\geq$ 0.25). Wood frog detection was also positively influenced by lower total dissolved solid values (<1000 mg/L TDS) and negatively influenced by very low catch-per-unit-effort values (<

0.01 CPUE). In contrast pesticides and ranavirus were poor predictors of wood frog presence, suggesting either the inability to avoid these stressors or resilience towards them. These results are consistent with previous studies regarding the importance of vegetation buffers and land use and cover, but highlight the effects of environmental factors at multiple scales on wood frog presence.

Tadpoles completed their larval development in both grassland and cropland sites. Body condition and N:L ratios were affected only by Gosner stage (GS); both were stable or slightly declined until metamorphic climax (GS 41-42), after which they declined greatly. There were, however, effects of environmental variables on tadpole and metamorph body mass. Besides Gosner stage, influential variables included total dissolved solids, proportion of pesticides detected, ammonia, and wetland surface area. Total dissolved solids and pesticide detection had marked negative effects on body mass at and above 600-700 mg/L TDS and 0.01 proportion of pesticides detected. Wetlands in the PPR are naturally saline, but the ionic composition is unique in that it is primarily sulfate ions and little research has investigated the effects of sulfates on tadpoles. Pesticide concentrations were lower than most lethal doses reported in the literature, but in the field setting where these tadpoles are simultaneously exposed to multiple stressors, it appears to have an impact on body mass. These results again emphasize the importance of multiple, interacting stressors on tadpole health as reduced mass at metamorphosis can have negative implications for survival and fecundity as an adult. I also observed unique neutrophils that warrant further research in wood frog hematology, especially with tadpoles at metamorphic climax.

It is clear that wood frogs can survive in these agricultural landscapes, but in order to maintain populations we need to monitor habitat characteristics at the water quality, wetland, and landscape scales. Agricultural activity can alter wood frog habitat at all of these scales, and all have implications for wood frog occupancy. Contaminant exposure may also affect life stages of the wood frog differently. Adult presence was not greatly influenced by pesticides, but tadpole and metamorph size was reduced which may have individual- and potentially population-level impacts. The results of these studies contribute new information to our understanding of wood frog ecology in a unique part of its North American range.

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2,4-D	Dichlorophenoxyacetic acid
a.e.	acid equivalent
ANOVA	Analysis of variance
ATV	*Ambystoma tigrinum* virus
AUC	Area under the receiver operating curve
Bd	*Batrachochytrium dendrobatidis*
BRT	Boosted regression tree(s)
CPUE	Catch-per-unit-effort
CV	Cross-validated in the context of BRT models; coefficient of variation in section 3.3.1
eDNA	Environmental DNA (deoxyribonucleic acid)
EPA	U.S. Environmental Protection Agency
FPWC	Federal policy on wetland conservation
GS	Gosner stage
HPT	Hypothalamic-pituitary-thyroid
HSD	Honestly significant difference
IPA	Isopropylamine
IUCN	International Union for Conservation of Nature
$LC50_{xxd}$	Lethal concentration required to cause 50% mortality of a test group in XX days
$LC50_{xxh}$	Lethal concentration required to cause 50% mortality of a test group in XX hours
MCPA	2-methyl-4-chlorophenoxyacetic acid
N:L	Neutrophil to lymphocyte
PCR	polymerase chain reaction
POEA	Polyethoxylated tallowamine
ppb	Parts per billion
PPR	Prairie pothole region
qPCR	quantitative PCR (polymerase chain reaction)
ROC	Receiver operating curve
RO_e	Reverse osmosis water enhanced with electrolytes
SMI	Scaled mass index

SVL	Snout-vent length
T3	Triiodothyronine
T4	Thyroxine
TDS	Total dissolved solids
USD	U.S. dollars
VES	Visual encounter survey

PREFACE

This thesis has been prepared in a manuscript style such that Chapter 1 serves as a general introduction to the thesis as a whole, Chapters 2 and 3 are data chapters focused on specific questions and prepared as manuscripts for subsequent publication in scientific journals, and Chapter 4 contains overall summaries and conclusions for the thesis. Thus, there is some overlap and repetition between the introduction and method sections of Chapters 2 and 3. Chapter 2 is currently being prepared for submission to *Ecological Applications* and Chapter 3 for *Journal of Herpetology*.

CHAPTER 1. A LITERATURE REVIEW: THE EFFECTS OF AGRICULTURAL LAND USE ON AMPHIBIANS

Declining amphibian populations throughout the world and increasing rates of decline indicate a pressing need to better understand the relationship between anthropogenic stressors and amphibian population health (Hopkins 2007, Kerby et al. 2010). Despite having survived four major extinction events in the past, amphibians are now at a particular risk in what is suggested to be the sixth mass extinction event (Wake and Vredenburg 2008). The causes of worldwide amphibian declines are varied and include habitat loss and degradation, environmental contamination, invasive species, emerging diseases, climate change, and overexploitation (Hopkins 2007, Wake and Vredenburg 2008, Blaustein et al. 2011, Lesbarrères et al. 2014). All faunal groups may be negatively affected by these factors, but amphibians are particularly susceptible because they require both aquatic and terrestrial habitats, tend to be herbivorous as young but carnivorous as adults, have permeable skin, are ectothermic, and typically have small ranges which implies the need for specific habitat requirements (Wake and Vredenburg 2008). Habitat loss is the primary threat to amphibians (Hopkins 2007, Mann et al. 2009) and while "degraded habitat" is often lumped in with "loss," it still allows populations to persist, at some level, amidst myriad challenges (Cushman 2006). The second greatest global threat to amphibians is pollution (Mann et al. 2009), and in vast landscapes converted to agriculture, wetlands become sinks for many contaminants. When this conversion occurs over a large scale (e.g., the Northern Great Plains) it becomes near impossible for aquatic wildlife to avoid the accompanying pollution. Disease, an emerging threat to amphibians, simultaneously impacts many amphibian species already affected by contaminants. While amphibians are continually faced with these multiple, interacting stressors *in situ*, the ways in which these interactions may affect amphibians are less studied than the effects of individual stressors (Blaustein et al. 2011, Battaglin et al. 2016).

1.1 WOOD FROGS

The wood frog (*Lithobates sylvaticus*) is one of the widest-ranging amphibian species of North America and is the only species known to exist north of the Arctic Circle (Martof and Humphries 1959, Martof 1970, Redmer and Trauth 2005). Its range extends diagonally across the continent from Alaska and across much of Canada and stretches southeast through the

Dakotas and into the Appalachian Mountains in Georgia, with small adjunct populations in Colorado and Wyoming (Martof and Humphries 1959, Martof 1970, Redmer and Trauth 2005, Powell et al. 2016). Aquatic habitat for wood frogs typically includes fishless seasonal and semi-permanent ponds, but may also include more permanent water bodies (Martof 1970, Trauth et al. 1989, Berven 1990, Hopey and Petranka 1994, Powell et al. 2016). Terrestrially, wood frogs can be found in a wide range of habitats including tundra, willow thickets and bogs, and temperate forests (Martof 1970, Redmer and Trauth 2005). The wood frog is also widely recognized as one of few vertebrate species that can survive sub-zero temperatures; as low as -6°C and for as long as two weeks at a time (Storey and Storey 1984, Costanzo and Lee 1994).

With its expansive range, it follows that life history and ecological traits of the wood frog exhibit wide geographic variation, and in the northern extents of its range these traits are often expressed in their extremes. For example, wood frogs of northern Manitoba and Saskatchewan are the smallest, averaging <40 mm in body length, but in the southern Appalachian Mountains they average in the 50-60 mm range and may reach record lengths > 80 mm (Martof and Humphries 1959, Martof 1970). Across their range, wood frogs are often one of the first amphibians to begin breeding in late winter and early spring, sometimes even when there is still ice on the water's surface, but the specific timing of breeding varies. Breeding may occur as early as January and February in the southern United States or as late as May and June in Alaska and northern Canada (Martof and Humphries 1959, Martof 1970, Trauth et al. 1989, Banta 1914, Redmer and Trauth 2005). Total time to complete metamorphosis also ranges from 65-130 days, although overwintering by tadpoles has also been suggested for populations in Alaska and Northern Canada (Martof 1970, Camp et al. 1990, Remder and Trauth 2005).

Being such a wide-ranging species, the wood frog may serve well as a local indicator of environmental stressors and effects for other frog species while maintaining comparability between studies. It is also considered a species of Least Concern by the IUCN (IUCN 2015) and its abundance in the Northern Great Plains makes it logistically preferable to study relative to other, less common species (e.g., northern leopard frog, *Lithobates pipiens*). In fact, it has already garnered popularity in contaminant research (e.g., Griffis-Kyle 2005, 2007, Storrs and Keisecker 2004, Burgett et al. 2007, Bergeron et al. 2011). In Saskatchewan, the wood frog may also act as an indicator amphibian species for the prairie biome, which is considered a "hot spot" of herpetological diversity in Canada despite historically poor protection and projected

increases in oil and gas exploration (Lesbarrères et al. 2014). While pollution is consistently described as a significant threat to amphibians (Hopkins 2007, Wake and Vredenburg 2008, Blaustein et al. 2011, Lesbarrères et al. 2014), wood frogs appear to persist in agriculture landscapes – thus begging the question, "how?" Further, the wood frog is identified as being especially susceptible to ranavirus (Hoverman et al. 2011). By defying the natural assumption that a species might decline noticeably in a landscape heavily converted for agriculture and being one of the most susceptible North American amphibians to ranavirus, the wood frog is an ideal study species to investigate the interactions between agricultural contaminants and disease, and has been similarly suggested as such by Miller et al. (2011) in their review of ranavirus ecopathology.

1.2 AGRICULTURAL CONTAMINANTS

Pollution is frequently identified as a significant contributor to global amphibian declines (Hopkins 2007, Mann et al. 2009, Egea-Serrano et al. 2012, Lesbarrères et al. 2014, Aldrich et al. 2016) and agricultural contaminants are important chemical stressors. In fact, Lesbarrères et al. (2014) posit agriculture as the greatest large-scale threat to Canadian herpetofauna. Prairie farmland, like that of southern Saskatchewan, contains hundreds of thousands of wetlands that provide habitat for a wide variety of fauna, including amphibians (Donald et al. 1999). However, organisms in these wetlands are faced with the threat of pollution from agricultural pesticides and fertilizers; the use of which has steadily increased since the 1960s and is projected to continue through 2050 (Puckett 1995, Matson et al. 1997, Donald et al. 1999, Tilman et al. 2001). Some of the most frequently detected pesticides in prairie pothole wetlands include the herbicides glyphosate, 2-methyl-4-chlorophenoxyacetic acid (MCPA), clopyralid, dichlorophenoxyacetic acid (2,4-D), bromoxynil, and dicamba (Donald et al. 2001, Messing et al. 2011). Albeit in lower concentrations than in wetlands in conventional farmland, some of these pesticides are also detected in wetlands in wildlife habitat free of direct pesticide use (Donald et al. 2001). In addition, fertilizers contribute to nitrogenous pollution and may come in the form of ammonia, nitrite, or nitrate. Ammonia and nitrite can be directly toxic to amphibians but they are both less stable in the environment than nitrate (Mann et al. 2009). Agricultural contaminants are an especially ubiquitous challenge to amphibians that live in the Canadian Prairie Pothole Region, and Saskatchewan in particular, where the majority of Canada's pesticides are applied (Statistics Canada 2008, Main et al. 2014).

Given their biphasic life history, amphibians are at risk from agricultural contaminant pollution both in freshwater and on land. Yet, despite widespread and increasing use, little information is available regarding pesticide application in Canada in either setting. Main et al. (2014) modeled the use and application of neonicotinoids (the most popular class of insecticides in Canada's prairie pothole region) based on standard application rates and crop land cover maps to estimate actual neonicotinoid application in Alberta, Saskatchewan, and Manitoba. Even fewer data are collected or available on pesticide concentrations in surface waters (e.g., neonicotinoids, Main et al. 2014). It is clear, though, that aquatic exposure to agricultural contaminants is an important exposure pathway and that concentrations in agricultural wetlands may exceed threshold guidelines and harm various aquatic fauna (Donald et al. 1999, Main et al. 2014). Further, guidelines are often based on exposure to individual contaminants and may underestimate their negative impacts by not accounting for possible synergistic effects from mixtures of contaminants (Donald 1999, Mann et al. 2009, Main et al. 2014). As many amphibians are explosive breeders, require water as developing tadpoles, and are most conspicuous when gathered together in the aquatic environment, it is both important and convenient to perform amphibian-contaminant studies during this phase.

Agricultural contaminants can have both direct and indirect effects on amphibians. Direct effects include mortality and various sub-lethal impacts (e.g., impacts on growth and metamorphosis, immunosuppression, deformity) and can be produced through ingestion of contaminants, ingestion of contaminated prey, or dermal uptake (Gibbons et al. 2015). While many studies investigate the direct, acute effects of individual pesticides, fewer studies investigate the chronic effects of pesticide mixtures (Relyea 2004, Carr and Patino 2011).

1.3 DIRECT EFFECTS OF CONTAMINANTS ON AMPHIBIANS

The study endpoints used to investigate direct effects of agricultural contaminants on amphibians are diverse. Mortality, growth, and development are frequently used and, for amphibians, metamorphosis also represents a critical life stage with several variables to use as endpoints. Other effects investigated in the literature include changes in behavior, physiological function, frequency of deformity, and impacts on the immune system.

1.3.1 Mortality and Survivorship

Mortality and changes in survivorship as a result of agricultural contamination are direct effects that could have population-level impacts. Environmentally realistic concentrations of contaminants likely do not reach lethal levels frequently, but there is clear evidence that there is the potential for contaminant-related death, especially when considering the possibility of synergistic effects from simultaneous exposure to multiple contaminants.

The exposure of five amphibian species, northern leopard frogs, green frogs (*L. clamitans*), bullfrogs (*L. catesbeianus*), American toads (*Bufo americanus*), and gray tree frogs (*Hyla versicolor*) to 1 mg/L of four common pesticides only caused significant mortality in 5% of the species-pesticide combinations, but 2 mg/L caused significant mortality in 35% of the same species-pesticide combinations (Relyea 2004). In this study, diazinon, malathion, and glyphosate caused mortality while carbaryl did not (Relyea 2004). Inasmuch as glyphosate-based products, such as Roundup Original® and Roundup WeatherMax®, have been shown to affect amphibian growth and development, they also have the potential to cause significant mortality (Howe et al. 2004, Lanctôt et al. 2014). This may be especially true for products containing the polyethoxylated tallowamine (POEA) surfactant (Howe et al. 2004). While the LC50 values determined for four species, northern leopard frogs, green frogs, wood frogs, and American toads, by Howe et al. (2004) are higher than the concentrations of glyphosate-based products typically found in the environment, the authors caution that amphibian larvae may take up glyphosate-based herbicides and additives via ingestion of plant material as well as through dermal absorption; a route that was not accounted for in their study. Similarly, while the predicted maximum environmental concentration of Roundup WeatherMax® (2.89 mg acid equivalent (a.e.)/L) killed all of the exposed wood frog tadpoles, the environmentally realistic concentration (0.21 mg a.e./L) did not affect survival (Lanctôt et al. 2014). Relyea and Jones (2009) tested 13 species of amphibians to determine $LC50_{96h}$ values of Roundup Original Max®, a POEA-containing glyphosate-based pesticide, and found that 2 mg a.e./L was necessary to elicit significant increases in mortality for seven of the nine frog species, including wood frogs. For these seven species then (wood frogs, leopard frogs, gray tree frogs, Cascades frogs - *Rana cascadae*, green frogs, American toads, and western toads - *Bufo boreas*), Roundup Original Max® may be classified as moderately toxic according to criteria set by the U. S. Fish and Wildlife Service and the U. S. Environmental Protection Agency (EPA, Relyea and Jones 2009).

Atrazine, another extremely popular pesticide, also increases mortality of amphibians at low, environmentally relevant concentrations. In fact, Storrs and Kiesecker (2004) found that the effects of atrazine on four frog species were expressed as nonmonotonic dose-response curves. For spring peepers (*Pseudacris crucifer*), American toads, and green frogs, 3 ppb significantly decreased survivorship in all but the late stage toad tadpoles (Gosner Stage 29-36, Storrs and Keisecker 2004). Only late stage wood frog tadpoles were tested in this study and while survivorship between low (3 ppb) and medium (30 ppb) atrazine concentrations was significantly different, there was no difference in survivorship between the low concentration and the control (Storrs and Keisecker 2004).

Nitrite and nitrate have also been investigated as sources of increased amphibian mortality and because these contaminants often enter aquatic ecosystems through contaminated runoff, it is important to use chronic testing regimes. When exposed to increasing concentrations of nitrite (0 to 6.1 mg/L NO_2-N) as embryos, wood frog and eastern tiger salamander survival to 34 d and 26 d, respectively, decreased significantly (Griffis-Kyle 2005). Individuals exposed to nitrite only as larvae had significantly greater survival than those exposed as both embryos and larvae (Griffis-Kyle 2005). Similarly, in another study, as ammonium nitrate exposure concentrations increased from 0 to 200 mg/L NO_3^-, wood frog survival over the course of one week decreased, and this effect was more pronounced for late stage larvae in the 50 and 100 mg/L concentrations (Burgett et al. 2007). Four northwestern species, Oregon spotted frog (*Rana pretiosa*), red-legged frog (*R. aurora*), western toad, and northwestern salamander (*Ambystoma gracile*) exposed to nitrate and nitrite for 15 days showed species-specific sensitivity for nitrate and unanimous sensitivity for nitrite (Marco et al. 1999). By day 15, Oregon spotted frogs and northwestern salamanders were the most sensitive to nitrate (i.e., greatest mortality) and had $LC50_{15d}$ values that are well within average nitrate concentrations found in regional crop lands (Marco et al. 1999). For all four species, nitrite $LC50_{15d}$ values were < 2 mg/L N-NO_2^- and fall below U.S. EPA water-quality criteria (Marco et al. 1999). In contrast to the results found by Marco et al. (1999) and Relyea (2004), however, Smith et al. (2011) exposed American toads and wood frogs to malathion, nitrate, and a combination of the two, and neither species displayed a significant decline in survivorship.

In studies with more natural community assemblages, the effects of contaminants on mortality become more complex. Relyea et al. (2005) exposed three tadpole species (gray tree

frogs, American toads, leopard frogs) to Roundup and malathion without predators, with predatory newts (*Notophthalmus viridescens*), or with predatory beetle larvae (*Dytiscus* sp.). Without predators or pesticides, survival for all three species was high (>70%), but exposing tadpoles to different combinations of predators and pesticides significantly changed their survival. Malathion alone did not reduce survival, and Roundup alone reduced survival significantly for the toad and leopard frogs (Relyea et al. 2005). Newts alone and beetles alone significantly decreased survivorship of all tadpole species (Relyea et al. 2005). Total tadpole survivorship when exposed to newts and malathion was not significantly different from newts alone, but when exposed to newts and Roundup, survivorship decreased (Relyea et al. 2005). Most interesting were the interactions between beetle presence and pesticide exposure. With beetles present, malathion significantly increased total tadpole survival indirectly by killing the beetle larvae, but adding Roundup had no effect on total tadpole survival (Relyea et al. 2005). This interaction is important to recognize and study because it better represents likely real-world scenarios. If a tadpole community exists in an environment where the majority of predators are beetles, then malathion may actually increase the tadpoles' survival, but if the majority of predators are newts then there may be no effect from malathion contamination (Relyea et al. 2005). These sorts of complex community and indirect effects are further discussed in Section 1.4.

1.3.2 Growth, Development, and Metamorphosis

The effects of contaminants on amphibian growth and development rates and time to metamorphosis are important endpoints to consider because delay in any of these processes may influence the individual organism's metamorphic and reproductive success which may then lead to population-level effects (Berven and Gill 1983, Relyea 2004, Todd et al. 2011, Smith et al. 2011). Generally, agricultural contaminants are found to impair growth and delay metamorphosis (reviewed by Mann et al. 2009). Given the complex interactions between an ever-growing list of contaminants, species-specific susceptibility, and environmental variables, however, this is hardly a rule of thumb. Laboratory studies are often used to examine the effects of agricultural contaminants on amphibian development, but they do not always corroborate with field studies (Mann et al. 2009, Lanctôt et al. 2014). Nevertheless, negative effects of pesticides and fertilizers on amphibian growth, development, and metamorphosis are well documented.

In the more straight-forward approach to studying the effects of agricultural contaminants on amphibians, researchers expose a species to a single contaminant. For example, Griffis-Kyle (2005) investigated the effects of nitrite on the ontogenetic development of eastern tiger salamanders (*Ambystoma tigrinum tigrinum*) and wood frogs in a laboratory setting and found that increasing nitrite concentrations had significant effects on hatching success and early larval growth. Greater nitrite concentrations reduced wood frog hatching success but not for eastern tiger salamanders (Griffis-Kyle 2005). A later study found that increasing nitrite exposure concentrations reduced growth and development rates for both species (Griffis-Kyle 2007). In the tiger salamander, increasing nitrite concentrations up to 2.1 mg/L NO_2-N lengthened the time it took for eggs to hatch, but at concentrations greater than 2.1 mg/L NO_2-N, eggs hatched faster (Griffis-Kyle 2007). Further, tiger salamander eggs exposed to greater nitrite concentrations hatched at lower development stages and were smaller in length. Wood frogs exposed to greater concentrations of nitrite also hatched at earlier development stages, although the time it took for eggs to hatch was not affected (Griffis-Kyle 2007). Larval wood frog growth was also inhibited as it took longer for tadpoles to reach metamorphosis in greater nitrite concentrations (Griffis-Kyle 2007). In a study of nitrate toxicity to the southern toad (*Bufo terrestris*), Edwards et al. (2006) raised tadpoles in two water 'types': spring water collected from an aquifer and tap water that had been purified through reverse-osmosis and then enhanced with electrolytes (RO_e). While nitrate had limited effects on southern toad growth and development, water type played a role in whether or not nitrate had any effects (Edwards et al. 2006). Tadpole growth rate was not affected by nitrate concentrations alone, but tadpoles raised in RO_e water were significantly larger (Edwards et al. 2006). There was no change in development rates as a result of either nitrate, water type, or their interaction, but time to metamorphosis was affected by the interaction. In RO_e water, greater nitrate concentration reduced the time it took for tadpoles to reach metamorphosis, whereas in the spring water, time to metamorphosis was significantly greater at the highest nitrate concentration (NO_3-N, Edwards et al. 2006). In spring water, tadpoles also did not grow as large. Edwards et al. (2006) make the case that not only is it important to consider the chemistry of the water used for laboratory studies, but also that chemical characteristics of spring water presents additional challenges to tadpole growth and development.

In more complex studies, researchers investigate the effects of multiple contaminants and/or their additives on more than one species. Relyea (2004) compared the effects of four common pesticides (diazinon, carbaryl, malathion, and glyphosate), individually and as mixtures, on five species of amphibians: northern leopard frogs, green frogs, bullfrogs, American toads, and gray tree frogs. Exposing any species to 1 mg/L of any individual pesticide rarely resulted in significant negative effects, but exposure to 2 mg/L of individual pesticides did begin to elicit species-specific patterns of reduced growth (Relyea 2004). Certain combinations of two pesticides, 1 mg/L of each pesticide, did cause greater reduction in growth relative to 2 mg/L of either pesticide alone for leopard frogs, green frogs, and bullfrogs (Relyea 2004). Overall, however, the pesticide combinations used in this study infrequently had greater negative impacts than 2 mg/L of either pesticide alone. In the field, thousands of agricultural contaminants are applied in various combinations, so while it is important to continue to study their interactive effects on amphibians, it may be possible to identify effects of certain combined pesticides simply by increasing their concentration (Relyea 2004).

Howe et al. (2004) and Lanctôt et al. (2014) investigated the effects of glyphosate-based pesticides on amphibians. Both studies included POEA, the surfactant found in many glyphosate-based products, as a contaminant and found reduced growth and development, particularly when exposed to glyphosate-based products that contain POEA or to POEA alone. However, Lanctôt et al. (2014) also concluded that, in their study, the effects of POEA on growth and development were not significantly greater than those caused by glyphosate in the form of isopropylamine (IPA) salt and suggest that POEA is not the only additive responsible for altered growth in amphibians. Furthermore, Lanctôt et al. (2014) compared their results to a similar study conducted in the field. In essence, the laboratory study overestimated the toxic effects of these glyphosate-based pesticides on amphibians and the field study revealed changes in macrophyte cover, phytoplankton biovolume, and zooplankton richness (Lanctôt et al. 2014). This case highlights the disparity between laboratory and field environments and that results from the lab may be a rather poor indication of what happens in the field.

Mixture studies often use combinations of pesticides but in an agricultural landscape, pesticides are also frequently found in tandem with nitrogenous fertilizers, and few studies have investigated the effects of a combination of pesticides and fertilizers. Smith et al. (2011) found that the effects of malathion and nitrate on American toads and wood frogs ranged from

synergistic to antagonistic and differed between the two species. For both species, malathion had greater individual effects than nitrate. Malathion exposure increased the time to metamorphosis for both American toads and wood frogs, but morphed toads had smaller average snout-vent length (SVL) and mass than control individuals while morphed wood frogs had larger average SVL and mass than control (Smith et al. 2011). Nitrate alone had no significant effect on either species' growth or development. Interestingly, for wood frogs, the addition of nitrate with malathion shortened the time to reach metamorphosis compared to wood frogs exposed solely to malathion at concentrations of 250 μg/L and 500 μg/L (Smith et al. 2011). This example further highlights the variability of effects that mixtures can have on different species.

1.3.3 Changes in Behavior and Physiological Function

Other effects of agricultural contaminants on amphibians include changes in behavior and physiology. Endpoints for these studies include changes in hormone levels of the hypothalamic-pituitary-thyroid axis (HPT), and changes in activity and bodily function. For example, when exposed to increasing levels of both nitrate and nitrite, northwestern salamanders and Oregon spotted frogs exhibited more infrequent feeding, weakened swimming ability, disequilibrium, and paralysis (Marco et al. 1999). All five test species, northwestern salamanders, Oregon spotted frogs, red-legged frogs, western toads, and Pacific tree frogs, showed these same abnormalities when exposed to increased concentrations of nitrite (Marco et al. 1999). Nitrate exposure has also been suggested to influence the HPT axis in amphibians. Whole-body T4 (thyroxine) concentrations in southern toads were significantly affected by nitrate exposure and by the type of water in which tadpoles were raised (RO_e or spring water, Edwards et al. 2006). When measured at the same life stage, in this case at forelimb emergence, whole-body T4 concentrations are anticipated to be the same in tadpoles despite exposure to different nitrate concentrations and being raised in different water types. However, T4 concentrations were higher in tadpoles raised in RO_e water than in spring water when exposed to 5 and 30mg/L NO_3-N (Edwards et al. 2006). Conversely, when exposed to a flux nitrate treatment (i.e., cycled between 0 and 30mg/L NO_3-N at each water change as opposed to a constant exposure concentration), whole body T4 concentrations were higher in tadpoles raised in spring water than those in RO_e water, albeit not significantly so (Edwards et al. 2006). The authors suggest that spring water contains other constituents that stress tadpoles which may increase the conversion of T4 to T3 (triiodothyronine) and the binding of T3 to its receptors. Thus, metamorphosis was

still achievable for seemingly T4-depressed tadpoles (Edwards et al. 2006). While this study does suggest that nitrate contamination can have a marked effect on the amphibian HPT axis, it is only one example. Various agricultural contaminants have been demonstrated to affect thyroid hormones, but the effects themselves are inconsistent and the mechanisms for changes in hormone concentrations are poorly understood (Carr and Patino 2011).

1.3.4 Deformities

Deformities are another consequence of amphibian exposure to contaminants and may present themselves in a variety of ways (e.g., missing vs. extra limbs or digits). While deformities may be alarming, especially if discovered on a large scale, they may occur naturally as a result of predator avoidance or other forms of physical trauma (Eaton et al. 2004). Wood frogs collected from five field sites with varying degrees of anthropogenic disturbance in Alberta and Saskatchewan exhibited a deformity rate of <2%, which is within assumed natural levels (Eaton et al. 2004). Deformities are also recognized as a result of parasitic infections (Johnson and Sutherland 2003, Eaton et al. 2004), of which the abundance and virulence may be influenced by agricultural pollution (Koprivnikar et al. 2006). Further, it has been suggested that agricultural contaminants may directly cause deformities on their own (Ouelett et al. 1997). Deformities can, in diverse ways, influence survivorship and population persistence. Limb and digital malformations may inhibit movement and predator avoidance and reduce individual survivorship (Ouelett et al. 1997). At the population level, male frogs exposed to certain agricultural contaminants (e.g., atrazine) may develop malformed testes and this likely influences reproductive success (Hayes et al. 2002). In any manifestation of deformity, external or internal, amphibian species may be susceptible to impacts at the population level.

1.4 INDIRECT EFFECTS OF CONTAMINANTS ON AMPHIBIANS

Indirect effects of contaminants on amphibians can occur from habitat modifications or changes in quality and/or quantity of prey/predators (Gibbons et al. 2015). Simple toxicity tests that investigate the effects of one or more contaminants on a single species fail to identify indirect effects, yet these are just as likely to occur, if not more so, in the environment. Studies that do investigate the indirect effects of contaminants on communities may still miss certain effects from trophic cascades if the tests are not long enough. Relyea and Diecks (2008) found that low concentrations of the insecticide malathion initiated a trophic cascade whose effects

persisted for well over a month. In several studies that have investigated the effects of contaminants on freshwater aquatic communities, common study organisms include zooplankton, phytoplankton, periphyton, predatory insects, predatory newts, and amphibian tadpoles.

The potential positive effect of malathion on tadpole survival in the presence of predatory beetle larvae described above contrasts with the results of Relyea and Diecks (2008) wherein malathion reduced zooplankton abundance that permitted a phytoplankton bloom and led to a decrease in periphyton abundance. This decrease in resources, in turn, did not affect wood frog larvae that morphed quickly, but leopard frog tadpoles did experience reduced growth and development and thus increased mortality as the environment desiccated before they could fully morph (Relyea and Diecks 2008). Additional evidence of indirect effects on tadpoles as a result of trophic cascades has been identified with atrazine and the insecticide endosulfan. Atrazine reduced wood frog tadpole development and growth as a result of a decrease in periphyton abundance, while endosulfan increased tadpole growth by reducing competition from chironomid larvae (Rohr and Crumrine 2005). Further emphasizing the contaminant-specific nature of community-level effects, Relyea (2005) found that two insecticides (carbaryl and malathion) had little negative effect on five tadpole species, and that carbaryl actually significantly increased total tadpole survival as a result of increased mortality of insect predators. One of the two studied herbicides, Roundup, resulted in almost 100% mortality in three tadpole species, yet while Roundup is designed to kill plants it had no direct effect on periphyton (Relyea 2005). Furthermore, Roundup indirectly increased the abundance of periphyton and decreased the abundance of predatory insects in response to the reduced abundance of tadpoles (Relyea 2005). In an effort to assess the scalability of the mitigating effects of macrophytes on contaminant exposure in aquatic ecosystems, Brogan and Relyea (2015) found that the presence of the macrophyte *Elodea canadensis* effectively eliminated any negative impacts of malathion. *E. canadensis* buffered zooplankton from the lethal effects of malathion and thus prevented a trophic cascade from ensuing (Brogan and Relyea 2015). Each of these studies emphasizes the complexity of effects that agricultural contaminants may have on freshwater aquatic communities. Pesticides may have negative or positive effects on tadpoles depending on the presence or absence of certain predators, and may be completely mitigated by the presence of macrophytes.

1.5 IMMUNITY

In light of emerging infectious diseases, the effect of agricultural contaminants on amphibian immune systems is a particularly important topic (Hopkins 2007, Wake and Vredenburg 2008, Lesbarrères et al. 2014). Recently, a hypothesis has emerged suggesting that the increased use of a novel type of pesticides, neonicotinoids, beginning in the 1990s is responsible for weakening the immune systems of various wildlife taxa which are now experiencing global disease-related declines, though current evidence is more correlative (Mason et al. 2013). Studies on the specific effects of agricultural contaminants on amphibian species' immune systems are few, but increasing.

Several endpoints may be used to assess amphibian immune health including white blood cell and phagocytic cell counts, assays of antibody titres, assays of phagocytic and lytic capacity, oxidative burst, and altered immune gene expression (Mann et al. 2009, Langerveld et al. 2009). In one study, leopard frogs exposed to a mixture of six pesticides experienced weakened immune systems (Christin et al. 2003). After 21 days of exposure to environmentally realistic concentrations of the pesticide mixture, frogs had reduced lymphocyte proliferation, a measure of immune response. After a three week recovery period (no pesticide exposure), lymphocyte proliferation in frogs that were exposed to a parasitic nematode species, *Rhabdias ranae*, recovered in all frogs except in those exposed to the highest pesticide concentration (Christin et al. 2003). Further, in the medium (21 μg/L atrazine, 0.56 μg/L metribuzin, 17 μg/L aldicarb, 0.15 μg/L dieldrin, 0.02 μg/L endosulfan, 0.33 μg/L lindane) and high exposure concentrations (210 μg/L atrazine, 5.6 μg/L metribuzin, 170 μg/L aldicarb, 1.5 μg/L dieldrin, 0.2 μg/L endosulfan, 3.3 μg/L lindane), 100% of tadpoles were infected with the parasite, compared to 80% in clean water and 70% in dimethyl sulfoxide, a common chemical used as a vehicle for three of the six pesticides used in the study (Christin et al. 2003).

One method to assess immune stress in vertebrates is the use of leukocyte (i.e. white blood cell) profiles because the ratio of neutrophils to lymphocytes is influenced by and closely related to levels of glucocorticoids, a class of stress hormones (Davis et al. 2008). While both cell types are involved in immune response, neutrophils are phagocytic cells that proliferate when an organism has an infection, experiences inflammation, or is otherwise stressed, whereas lymphocytes play a role in the production of immunoglobin and help adjust the overall immune response (Davis et al. 2008). The relationship with glucocorticoids exists because the release of

these hormones causes lymphocytes to move from the blood stream to various other organs and neutrophils to move from bone marrow to the blood stream such that an increase in the neutrophil:lymphocyte (N:L) ratio in the blood stream reflects an increase in glucocorticoid levels (Davis et al. 2008). In one study, northern leopard frogs with higher loads of parasites (*Hepatozoon* spp.) had elevated N:L ratios, but the authors did not find a similar relationship between N:L ratios and pesticide concentrations (Shutler and Marcogliese 2011). The effects that agricultural contaminants may have on N:L ratios in amphibians are inconsistent. In some studies the N:L ratios increase in amphibians exposed to agricultural contaminants, where other studies show no effect (Shutler and Marcogliese 2011). Shutler and Marcogliese (2011) suggest that the lack of data on amphibian leukocyte profiles and their susceptibility to be affected by a myriad of natural and anthropogenic factors leads to an inability to conclude on the overall effects of pesticides and parasites on leukocyte profiles.

1.6 RANAVIRUS

Ranavirus is of particular concern for North America, where the majority of documented world-wide ranavirus mortality events have occurred (Miller et al. 2011). In an investigation of 44 amphibian mortality events in the USA, Green et al. (2002) found that the majority were caused, either completely or partially, by ranavirus. The reasons for the recent emergence of mass amphibian mortality events caused by ranaviruses remain poorly understood but are thought to be related to anthropogenic disruption (Miller et al. 2011).

Generally, tadpoles and metamorphosing tadpoles are the most susceptible life stages to ranavirus infection (Miller et al. 2011). Die-offs may take place over the course of a few days to months and, as most wetlands are not frequently monitored, by the time a field report is made the only evidence is mortality and the symptoms are no longer evident (Miller et al. 2011). Symptoms of ranavirus infection in amphibian larvae include poor swimming and buoyancy, lethargy, weight loss, erythema, cutaneous polyps, intracoelomic lesions, internal hemorrhaging, and swollen limbs (Gray et al. 2009, Miller et al. 2011). These symptoms are not always present and vary according to life stage and infection level (Gray et al. 2009). Transmission of ranavirus can occur through exposure to contaminated water or sediment, direct contact with other infected individuals, or the consumption of tissues infected with ranavirus (Miller et al. 2011). Ranavirus can resist inactivation for up to 80 days and, in soil, resistance is prolonged further as temperatures decline (Nazir et al. 2012). This suggests that ranavirus may be able to withstand

deterioration from year to year if the conditions are right, thus providing the ability to re-infect a new cohort of amphibians each year. Other possible reservoirs include post-metamorphic amphibians, aquatic larvae that take > 1 year to metamorphose, highly aquatic adult amphibians, and a variety of fish and reptiles (Gray et al. 2009).

The net effects of the relationships between ranavirus, amphibians, and pesticides are also variable. Forson and Storfer (2006b) found that moderate concentrations of atrazine decreased infectivity of the *Ambystoma tigrinum* virus (ATV) and mortality in the long-toed salamander (*Ambystoma macrodactylum*). In contrast, atrazine increased immunosuppression and susceptibility to ATV in tiger salamanders (Forson and Storfer 2006a). Reasons for this discrepancy are unclear. In laboratory tests of 19 amphibian species, the wood frog was one of the most susceptible species to ranavirus isolates (Hoverman et al. 2011). However, the wood frogs used in this study were from Tennessee, USA and, given the wood frog's wide range, it would benefit ranavirus research to investigate interactions between ranavirus and this species in additional locations. This is especially true since ranavirus has rarely been reported in Saskatchewan wood frogs despite being identified as the cause of tiger salamander mortality in Regina (Bollinger et al. 1999, Schock et al. 2008). The high percentage of land used for agriculture in the prairie pothole region around Saskatoon also provides suitable study sites to investigate the interactions between pesticides and ranavirus. Many reports of ranavirus outbreaks are made after die-offs are observed. However, ranavirus can be detected using environmental-DNA (eDNA) techniques (Hall et al. 2016, Pierson and Horner 2016) that involve the isolation of DNA from samples taken from the environment rather than the organism (e.g., water, soil, air). The detection of ranavirus eDNA in natural waterbodies is a novel area of research, but most recently Hall et al. (2016) found a strong relationship between eDNA ranavirus titres in the water and titres in larval wood frogs. Ranavirus eDNA was present in the water at increased levels just before a die-off and remained present afterwards (Hall et al. 2016).

In a recent study, Pochini and Hoverman (2017) examined the effects of ranavirus exposure on pesticide toxicity and the effects of pesticide exposure on ranavirus susceptibility in wood frogs. Exposure to ranavirus significantly increased the toxicity of both tested pesticides, such that the $LC50_{48h}$ value decreased by 72% for carbaryl and by 55% for thiamethoxam (Pochini and Hoverman 2017). The effects of pesticide exposure on the tadpoles' susceptibility to ranavirus were more complex. Pre-exposure to carbaryl caused tadpoles to die from ranavirus

quicker if they were challenged with ranavirus immediately after pesticide exposure, but pre-exposure to thiamethoxam did not (Pochini and Hoverman 2017). Whether tadpoles were challenged with ranavirus either immediately after the pesticide exposure or after spending 14 days in clean water, the infection rate of tadpoles was 100% for both pesticides (Pochini and Hoverman 2017). This study's results are particularly interesting because they illustrate that one stressor can hinder the wood frog immune system enough to increase the risk of a second stressor, but also that order and timing of exposure to these stressors influences their risk. In tadpoles infected with ranavirus, the LC50 values of carbaryl and thiamethoxam decreased to values reported in surface water (Pochini and Hoverman 2017). Tadpoles which were given time to recover from pesticide exposure were less quick to die from ranavirus than those exposed to the virus immediately; however, there were also no differences in viral load upon death between immediate and delayed ranavirus exposure. When ranavirus-free tadpoles were exposed to an infected individual that had also been exposed to pesticides, all 'naïve' tadpoles became infected but in the carbaryl exposure, naïve tadpoles' viral loads were lower than the infected 'focal' tadpole (Pochini and Hoverman 2017). This study, being one of few that examines the interactive effects of agriculture and ranavirus on amphibian immunity, lays the groundwork for future research.

1.7 OBJECTIVES AND HYPOTHESES

The primary goal of this study was to investigate the effects of agricultural land use on the presence and health of wood frogs in the Saskatchewan Prairie Pothole Region. This was accomplished in two main objectives:

1) Assess wood frog presence, using eDNA, at wetlands across a gradient of agricultural intensity (grassland vs. cropland sites), and relate this abundance to a variety of natural and anthropogenic factors. Follow up analyses looked for differences in habitat variables among sites.
2) Evaluate wood frog tadpole and metamorph health using morphometrics and blood neutrophil to lymphocyte ratios. Tadpoles and metamorphs were collected from wetlands across the agricultural gradient and health responses were modeled against natural and anthropogenic variables to explore potential relationships.

I hypothesized that wood frog populations are present in agricultural environments, and predicted that there would be no significant difference in wood frog presence or abundance from

wetlands that vary in agricultural intensity. However, I hypothesized that wood frog health is compromised by agricultural contaminants and predicted that tadpoles originating from cropland wetlands would exhibit reduced health in the form of smaller body condition or mass and elevated blood N:L ratios compared with tadpoles from grassland locations.

CHAPTER 2. ASSESSING THE EFFECTS OF AGRICULTURAL LAND USE ON THE PRESENCE OF WOOD FROGS (*LITHOBATES SYLVATICUS*) IN CENTRAL SASKATCHEWAN, CANADA

PREFACE

The primary aim of this study was to investigate the effects of water quality (including agricultural contaminants), wetland habitat, and land use variables on the presence of wood frogs in Prairie Pothole wetlands of central Saskatchewan, Canada. This chapter's focus is on the potential for agricultural activities to have population-level effects on wood frog occupancy of wetland habitat. The authors and contributions of this chapter are as follows: Gabrielle E. Ruso – designed the project, collected field and lab data, analyzed the data, and drafted the manuscript; Dr. Christy A. Morrissey – provided guidance for project design and statistical analyses, managed and funded pesticide sample collection, and provided comments and edits of the manuscript; Dr. Natacha S. Hogan – provided guidance for project design and comments and edits of the manuscript; Dr. Claudia Sheedy – processed pesticide samples; Melanie J. Gallant – cultured ranavirus for positive controls; and Dr. Timothy D. Jardine – provided guidance for project design and statistical analyses, comments and edits of the manuscript, and provided research funds.

2.1 INTRODUCTION

Wetland ecosystems across the globe are important to both human society and wildlife communities but are faced with multifaceted challenges associated with degradation. Wetlands provide ecosystem services such as regulating local and regional water availability, flood abatement, water quality improvement, groundwater recharge, and carbon sequestration (Zedler 2003, Russi et al. 2013). They also provide critical habitat for many organisms including invertebrates (Wrubleski and Ross 2011), birds (Kantrud and Stewart 1977), and amphibians (Collins and Storfer 2003). Costanza et al. (2014) calculated that wetland ecosystems provide services that equate to $26.4 trillion (USD) in 2011, down from an estimated $36.2 trillion (USD) in 1997 due to loss of global wetland area. Wetland ecosystems have been drained, or otherwise altered for millennia, primarily due to agriculture, but also urbanization and industrial development (Davidson 2014, Gardner et al. 2015). It is estimated that, during the 20th century

alone, the global land area of wetlands declined by 64-71% with inland wetlands facing greater loss than coastal wetlands (Davidson 2014, Gardner et al. 2015).

One global region where degradation of wetland ecosystems due to agriculture is especially prevalent is the Prairie Pothole Region (PPR) of North America. The PPR extends from Alberta in Canada, south and east through Saskatchewan, Manitoba, the Dakotas, Minnesota, and Iowa (Van Meter and Basu 2015). The PPR is critically important to native wildlife that inhabit this region and to migratory birds that use these prairie wetlands as stopover points or as breeding grounds (Kantrud and Stewart 1977, Fairbairn and Dinsmore 2001). However, the intense agriculture activity in the PPR has resulted in a heavily modified ecosystem in which wetlands may be altered both physically and chemically and these modifications can have important impacts on habitat quality for wildlife species.

Ways in which wetlands can be modified physically include drainage, consolidation, and removal of vegetation buffers. Consolidation occurs when wetlands in the upper reaches of a basin are drained into fewer, larger wetlands (Anteau et al. 2016). In Saskatchewan alone, Huel (2000) estimated that 40% of its wetlands have been lost due primarily to drainage. Consolidated wetlands tend to be larger, more permanent, and do not experience changes in water level to coincide with seasonal climate variations (McCauley et al. 2015, Anteau et al. 2016, McCauley et al. 2016). Consequently, these wetlands are likely to become less suitable as habitat for native species. Altered hydrological regimes deter birds (McCauley et al. 2016) and deeper, more permanent wetlands favor the establishment of fish populations which alters invertebrate communities important to native bird and amphibian diets (McCauley et al. 2015, Anteau et al. 2016). Vegetation buffers around wetlands are biologically important for many faunal groups, but particularly herpetofauna (Semlitsch and Bodie 2003), and recent evidence suggests that these buffers may enable reduced pesticide exposure for this sensitive taxon (Main et al. 2017, Swanson et al. 2018).

Nutrient and pesticide contamination in agriculturally altered wetland habitat can also impact amphibians. Field studies have reported pesticides in frog tissues (Smalling et al. 2015, Battaglin et al. 2016) and lab studies have demonstrated negative effects of nutrient and pesticide contamination on amphibian survival and growth (Relyea 2004, Griffis-Kyle 2005). The ability of vegetative buffer zones to improve water quality is variable (Mander et al. 2017) but evidence exists for their removal of nutrients and pesticides. In many instances, the focus of wetland and

riparian buffer zones and their ability to remove nutrients (nitrogen and phosphorus) from water is in relation to the downstream effects of these elevated nutrient concentrations (e.g., the Mississippi River Delta, Mitsch et al. 2001, Mander et al. 2017). However, vegetation buffers also reduce nitrogen and phosphorus contamination into wetlands from agricultural runoff (Haoukos et al. 2016) and lower neonicotinoid concentrations in wetland water (Main et al. 2017).

These physical and chemical modes of wetland habitat alteration are each represented in the agriculturally impacted wetlands of the PPR in Saskatchewan, Canada. Wetlands provide habitat for many wildlife species in the PPR, but industrialized agriculture, considered to be one of the greatest threats to herpetofauna (Lesbarréres et al. 2014), inherently fragments natural habitat, and reduces habitat quality (Porej et al. 2004). This is particularly true in Saskatchewan where wetland loss is of heightened concern both environmentally and socio-economically (Pattison-Williams et al. 2018) and for wood frogs (*Lithobates sylvaticus*) wherein evidence suggests that forest cover in the greater landscape is critically important in maintaining suitable habitat overall (Porej et al. 2004). Despite agricultural impacts, some amphibian species, like the wood frog, are still found in these ecosystems. Wood frogs breed in seasonal and semi-permanent wetlands but as adults they may spend time in a variety of habitats including woodlands, forests, meadows, and in the vegetation buffers around wetlands (Redmer and Trauth 2005, Swanson et al. 2018). Thus, the presence and persistence of wood frogs across the PPR landscape is likely influenced by habitat suitability at large (e.g., land use) and small (e.g. water quality) scales (Marsh and Trenham 2001, Porej et al. 2004, Hayes et al. 2010, Battaglin et al. 2016). In addition to these multi-scaled factors of habitat suitability, amphibians also face the challenges of parasitic infections and emerging infectious diseases with potentially impaired immune systems as the result of exposure to agricultural contaminants (Rohr et al. 2008, Hayes et al. 2010, Mason et al. 2013, Pochini and Hoverman 2017). In lab studies, wood frogs have shown greater susceptibility to ranavirus compared to other anurans and this further emphasizes the importance of assessing multiple, interacting factors on the species' presence in altered environments (Hayes et al. 2010, Hoverman et al. 2011). Understanding how these variables may influence wood frog presence in an agricultural landscape may provide important insights for our understanding of the species' ecology and for management decisions.

To assess the impacts that agriculture may have on wood frog presence in PPR wetland habitat of central Saskatchewan, I measured multiple variables across grassland and cropland sites pertaining to ranavirus presence, water and wetland habitat quality, pesticide concentrations, and land use. I then measured differences in the frequency of wood frog presence between survey methods (traditional vs. eDNA) and across sites, and used Boosted Regression Tree (BRT) modelling to determine how each variable influenced wood frog presence or absence in this study region.

2.2 METHODS

2.2.1 Study Area and Wetland Selection

Between May and July of 2017 and 2018, I visited wetlands at five sites, two grassland and three agriculture-dominant (cropland), near Saskatoon, SK. These sites were chosen because of their accessibility and historical use by other researchers in the past (Stanton et al. 2016, Michelson et al. 2018). In 2017, four wetlands were near Allan, SK (grassland, 51.6260 N, -105.9717 W), five at the St. Denis National Wildlife Area (grassland, 52.2153 N, -106.0770 W), six near Burr, SK (cropland, 51.9809 N, -105.0774 W), six near Colonsay, SK (cropland, 52.0264 N, -105.9217 W), and five near Humboldt, SK (cropland, 52.1993 N, -105.2883 W), totaling 26 wetlands. At Humboldt, approximately half of the site was acquired by Ducks Unlimited in 2016 and has undergone some habitat restoration (R Clark 2018, pers. comm.). In 2018, I increased the total number of observed wetlands to 71 with six at Allan, 14 at St. Denis, 11 at Burr, 18 at Colonsay, and 22 at Humboldt, although this total was reduced to 65 by the end of the field season due to wetland desiccation. Prior to the 2018 field season, I created circular zones in ArcMap (ESRI 2017) within which I surveyed all wetlands for wood frog presence. At Allan and St. Denis, I created single circles with radii of 0.4 km as they contained all the original wetlands from 2017. At the remaining sites I had to create two circles of 0.28 km radii each to contain the original 2017 wetlands and simultaneously cover the same total area as for Allan and St. Denis. One exception to this is the sixth wetland at Burr in 2017. I could not create two circular zones that could contain the original five wetlands and this additional one, but as it was close to the 2018 search areas I still included it.

In June and July of 2017, I performed visual encounter surveys (VES) for wood frog presence, dipnet surveys for tadpoles, measured water quality, and habitat characterization at all

wetlands. In 2018, I performed VES at all wetlands in the early spring (May). In the summer (June and July), I measured water quality and characterized wetland habitat at all of the original wetlands surveyed in 2017. In addition, I randomly selected up to five more wetlands per site to measure water quality and perform habitat characterizations, and for all wetlands at which I saw or heard wood frogs I performed dipnet surveys for tadpoles. Finally, at all wetlands I determined surrounding land use in ArcMap and in both years I collected water samples for environmental DNA (eDNA) to determine absence or presence of ranavirus and wood frogs (Hall et al. 2016). More detailed methods for eDNA sampling, VES and dipnet surveys, water quality, habitat characterization, and land use determination follow below and descriptions of model variables are provided in Table A-1.

All samples were collected under a Saskatchewan Ministry of Environment research permit (#17FW204), an Environment and Climate Change Canada National Wildlife Area access permit (#2017-072), and with University of Saskatchewan Animal Use Protocol approval (#20170055).

2.2.2 Environmental DNA Collection and Processing

Environmental DNA (eDNA) is a method used to detect occurrence of target species' DNA in environmental media, oftentimes water samples, and is especially useful for detecting cryptic and/or rare species like many amphibians (Goldberg et al. 2018). eDNA, however, is not perfect and detection probability can be influenced by several variables. Examples of factors which may influence detection probability include individual production rates of shed DNA material (e.g., skin cells), degradation rates of DNA which may be influenced by water chemistry, microbial activity, and other environmental factors, the likelihood of DNA to bind to sediment, and the ability of DNA to either move through the system (streams) or remain more sedentary (lakes; Goldberg et al. 2016, 2018). Given these factors, it is estimated that eDNA remains detectable between 1 day and 8 weeks after being shed from the organism (see Goldberg et al. 2016). To deal with these factors, researchers should tailor studies to account for target species ecology (i.e., when the organisms are most likely to be in the water based on life history), characteristics of the surveyed environment, and potential for field and lab processing contamination (Goldberg et al. 2016, 2018).

In summer 2017, spring 2018, and summer 2018 I collected three 250 ml water samples from each wetland for eDNA. In 2017 all water samples were filtered through 0.4 μm cellulose

nitrate filters in the field and the filters were preserved in 2 ml tubes filled with 100% ethanol. In 2018, all water samples were kept at 4°C and filtered in the lab within three days of collection. DNA was extracted from the filters using a modified method with the DNeasy blood and tissue kit (Qiagen, Inc.). DNA samples were diluted to 50 ng/µl before being tested for either ranavirus or wood frog presence using qPCR on 96-well plates. To test for the presence of ranavirus I used the Taqman assay developed by Picco et al. (2007) which targets a region of the major capsid protein, and to test for the presence of wood frogs I used a Taqman assay developed by Dysthe et al. (pers. comm.) which targets the cytochrome b gene. The reaction methods used for qPCR differed for each test. Ranavirus reactions were comprised of 10 µl Taqman Environmental Master Mix (Applied Biosystems, Foster City, California, USA), 2 µl of sample, 300 nM forward primer, 900 nM reverse primer, and 250 nM fluorescent probe, totaling 20 µl. Wood frog reactions included 7.5 µl Taqman Environmental Master Mix, 4 µl of sample, 900 nM forward primer, 300 nM reverse primer, and 250 nM fluorescent probe, totaling 15 µl. Every sample (n = 3 per wetland) was run in triplicate for 45 cycles and each 96-well plate included positive and negative controls. Prior to running field samples, I tested the primer assays using serial dilutions of positive controls with known DNA quantities and determined efficiencies of 99.42% for the ranavirus assay and 104.45% for the wood frog assay. For diagnosis of wood frog and ranavirus presence/absence, if two or three replicates of a single sample amplified during qPCR, the sample was scored as a positive (Tables A-2, A-3, A-4). If one well amplified, the sample was rerun in triplicate and if one or more wells amplified then it was scored as a positive. If none of the three replicates amplified, then the sample was scored as negative. If any of the three samples for a wetland were scored as positive in qPCR then the wetland itself was also scored positive for either ranavirus or wood frog presence. In 2018, I collected field blanks, one for approximately every fourth wetland, and all of these (n = 25) scored negative.

2.2.3 Traditional Surveys: Visual Encounter and Dipnet Surveys

To perform VES, I walked the perimeter of each wetland and visually and audibly surveyed for wood frogs. In 2017, I surveyed in the summer and thus was only looking for juvenile and adult frogs but in 2018 I conducted the surveys in spring and also looked for egg masses. In 2017, I surveyed an initial 24 wetlands for tadpoles using dipnet sweeps. After making few positive observations, I then surveyed an additional 1-3 wetlands at each site, totaling 37 wetlands surveyed for wood frog presence. Of these additional surveyed wetlands,

two contained tadpoles, thus establishing 26 wetlands as the original set described above (Section 2.2.1). In spring 2018 I performed VES at all 71 wetlands and for wetlands in which wood frog presence was observed, I also performed dipnet surveys for tadpoles conducted later in the summer.

I performed all of the dipnet sweeps in order to minimize observer bias, generally following the methods of Shulse et al. (2010) to establish catch per unit effort (CPUE). I made one dipnet sweep for every 25 m^2 surface area, with a minimum of 5 and maximum of 20 sweeps per wetland. I estimated the surface area of each wetland *a priori* using the ArcMap World Imagery basemap (ESRI 2017) and drawing polygons around each wetland to then calculate the number of sweeps to perform. Some of these wetland surface area estimates were initially ground-truthed and found to be accurate except for very large, recently flooded wetlands. Further, most wetlands were large enough to require the maximum number of sweeps and, thus, especially accurate surface area estimates were of lesser concern. I conducted sweeps by placing the bottom edge of a D-net at the bottom of the wetland approximately 1 m away and then swiftly pulling the net towards me. I generally made sweeps in the edge habitat of wetlands throughout as much of the perimeter as possible. I collected all captured tadpoles in a 5 gallon bucket for subsequent processing. I combined the results of VES and dipnet surveys to identify whether I did or did not observe any stage of wood frogs at a wetland. Together these are defined as "traditional" survey methods, as opposed to the aforementioned eDNA methods.

2.2.4 Water Chemistry Analyses

To measure water chemistry variables at each wetland, I used a sonde (YSI model exo^2) to measure dissolved oxygen (mg/L), pH, total dissolved solids (TDS mg/L), and turbidity (FNU). I also collected water samples for nutrient and pesticide measurements. I collected 500 ml of unfiltered water for total nitrogen and phosphorus (mg/L; 250 ml in 2018), 50 ml of filtered water (0.45µm filter, Sarstedt) for dissolved nitrogen (nitrate, N mg/L), dissolved phosphorus (phosphate, P mg/L), and ammonia (N mg/L), and filtered up to 500 ml of water with a glass fiber filter (Whatcom GF/F) for chlorophyll a (µg/L) measurements. During 20–24 June, 2017 and 18–22 June 2018, I collected 1L of unfiltered water to use for pesticide analysis. In 2017, I collected pesticide samples from almost all wetlands (n = 24) and in 2018 from a subset of wetlands (n = 23). The total and dissolved nutrient water samples were kept frozen at -

20°C, the chlorophyll a sample filters were folded, wrapped in foil, and frozen, and the pesticide samples were kept refrigerated at 4°C until analysis.

In the lab, I measured dissolved nitrogen, dissolved phosphorus, and ammonia using a YSI photometer, following the manufacturer protocols. I also measured chlorophyll a in samples on a Trilogy fluorometer (Turner Designs) following extraction in hot ethanol. The total nitrogen and phosphorus samples were analyzed following methods described in Abirhire et al. (2016). The pesticide samples were analyzed for neonicotinoid concentrations following Main et al. (2014) and were shipped to Dr. Claudia Sheedy's lab at Agriculture and Agri-Food Canada in Lethbridge, Alberta to determine concentrations of a suite of pesticides using gas chromatography–mass spectrometry. In 2017 and 2018, the water samples were screened for a total of 166 and 172 pesticides, respectively. In both years, these included four acaricides, one bactericide, 31 fungicides, one growth regulator, 62 herbicides, 60 insecticides (including one synergist), one nematicide, and four neonicotinoids. Ten pesticides were screened for by Dr. John Headley's lab at the National Hydrology Research Centre, including the four neonicotinoids (imidacloprid, thiamethoxam, clothianidin, and acetamiprid) in both years and three diamides (chlorantraniliprole, cyantraniliprole, and flubendiamide) and three additional insecticides (flonicamid, flupyradifurone, and sulfoxaflor) in 2018 only (Table A-5). For modeling purposes (see Section 2.2.7.2), I summarized pesticides in two ways: (1) as the proportion of pesticides detected out of the total number screened for (i.e., "pesticide detections" or "proportion pesticides detected"), and (2) as the sum concentration of detected pesticides. In using the sum concentration (effectively concentration addition) of all detected pesticides that include both herbicides and insecticides, I violate the assumptions that all detected pesticides act through similar modes of action and have equal toxicity. Despite this, I used this method for two reasons: 1) I observed very low concentrations of any individual pesticide (mean = 0.20 μg/L, maximum = 3.21 μg/L), with very low likelihood that individual compounds would have particularly strong effects on wood frogs (Relyea 2005, Johnasson et al. 2006, Robinson et al. 2017, Lee-Jenkins and Robinson 2018), and 2) there is evidence elsewhere for the use of the concentration addition method even when a variety of pesticides are represented (Junghans et al. 2006). I did not use a toxic units approach because of the inability to find LC_{50} or EC_{50} values for wood frogs for every detected pesticide and I wanted to avoid making assumptions across

taxa (e.g., fish to frogs). While this may overestimate the toxicity of mixtures of dissimilar pesticides, I preferred to take a conservative, precautionary approach.

2.2.5 Wetland Habitat Characterization

I generally followed the rapid wetland assessment protocol established by Main et al. (2015) in which I quantitatively and qualitatively assessed wetland habitat characteristics including hydrogeomorphology, wetland vegetation cover, and overall wetland classification (Table A-1). At each wetland I recorded latitude and longitude (WGS84), determined basin fill code, wetland situation, connectivity to other wetlands, wetland vegetation cover code, wetland classification, and crop type or surrounding land use, bison or cow presence or absence, fish presence or absence, estimated percent of the wetland surface that was algae cover, and took width measurements at each cardinal direction of wet meadow and shallow marsh. I used ArcMap (ESRI 2017) to estimate surface area and distance to the nearest road.

2.2.6 Land Use Determination

The radius used to calculate proportional land use around study wetlands was determined by considering wood frog natural history. In one study in Minnesota, the home range of wood frogs was estimated at 64 m^2 (77 yd^2, Bellis 1965). This translates to a circle with a radius of approximately 4.5 m. In Virginia, Berven and Grudzien (1990) investigated the genetic population structure of wood frogs. They concluded that adult wood frogs are highly philopatric and that the radius of genetic neighborhoods is about 1000 m (Berven and Grudzien 1990). However, due to the variation in distances traveled by dispersing frogs, the authors note that this value is likely an overestimation. To ensure that land-use intensity matched the maximum potential area experienced or used by wood frogs, I chose this latter radius (also see Porej et al. 2004). I used the raster data "Annual Crop Inventory" land use file for 2016 produced by Agriculture and Agri-Food Canada (AAFC 2016) and the Tabulate Area 2 tool from the Spatial Analyst Supplemental Tools toolbox (Noman 2013) to classify and calculate the area of land used as either crops (barley + oats + spring wheat + canola and rapeseed + lentils + soybeans + peas), pasture and forage, natural (coniferous + broadleaf + mixedwood forests + shrubland + grassland and prairies), urban and developed (includes roads), exposed and barren land, or water and wetlands (Table A-1). The "Annual Crop Inventory" raster file has a resolution of 30 m with

Saskatchewan-specific accuracies of 92.26% for crop classes and 69.90% for non-crop land cover classes.

2.2.7 Statistical Analyses

To determine how potential habitat for wood frogs varied between grassland and cropland sites, I tested all environmental variables for differences among sites. I used ANOVAs and Tukey's HSD tests when data were normally distributed and homoscedastic, with or without log-transformation, and Kruskal-Wallis and Dunn's tests when they were not. Post-hoc comparisons were made with p-values adjusted using the Holm's method for the Dunn's tests. Factor variables were tested with Fisher's exact tests and pairwise post-hoc tests when necessary.

2.2.7.1 Wood Frog Presence/Absence

Statistical analyses for wood frog presence/absence were conducted with Chi-squared tests and Fisher's exact tests where applicable. I used these tests to identify differences in wood frog detection success between survey methods ("traditional" vs. eDNA), and also to identify differences in eDNA detection success for wood frogs and ranavirus between seasons, years, and among sites. Although eDNA was collected in both spring and summer of 2018, statistical comparisons between years were only made with summer data to maintain similarity between the sample sets. For comparisons between detection success of traditional and eDNA survey methods and for assessing proportion of wood frog presence between sites, I split analyses by year because only some wetlands were surveyed in both years, not all.

2.2.7.2 Factors Influencing Wood Frog Presence

To model the influence of water quality, wetland habitat, and land use variables on wood frog presence, I used BRTs with the eDNA results. Because some wetlands dried up between spring and summer in 2018, the total number of wetlands used in the BRT analyses was reduced to 71 with 26 in 2017 and 45 in 2018. The reduced number of wetlands in 2018, compared to the total number of wetlands from which eDNA samples were collected, is due to the fact that not all wetlands were also surveyed for water quality and wetland habitat data and, given the small sample size to begin with, I wanted to keep the data set as complete as possible. Further, due to extremely high correlations ($r > 0.9$) between total dissolved solids and conductivity, and between dissolved and total phosphorus, I removed conductivity and total phosphorus from the

models. Although BRTs are amenable to correlated variables, their inclusion can affect the relative influence value and partial dependence plots of variables (Soykan et al. 2014). Summary statistics were calculated in Microsoft Excel and all additional analyses were conducted in program R v. 3.5.1 (R Core Team 2018) with $\alpha = 0.05$.

Boosted regression trees are a relatively new modeling technique gaining traction in ecological applications (De'ath 2007, Elith et al. 2008, Sokyan et al. 2014, Main et al. 2015). In essence, BRT are a combination of regression trees and machine learning such that a single BRT model contains hundreds to thousands of trees to describe an ecological phenomenon (De'ath 2007, Elith et al. 2008). In more traditional modeling approaches, one finds a 'best fit' model, but with the BRT approach, many trees are combined to best explain the data such that after the first tree is fit, the second tree works on the residuals of the first model, and so on (Elith et al. 2008). In this way, BRT identify general rules of thumb and then sequentially work to explain remaining variation (Elith et al. 2008). BRT are particularly useful in ecological applications because they can handle a variety of explanatory variables with different scales, and are unaffected by outliers or missing data (Elith et al. 2008).

When working with BRT, there are several components that one may alter to optimize the model including the learning rate, tree complexity, and bag fraction (De'ath 2007, Elith et al. 2008). The learning rate reflects the 'weight' or contribution that each subsequent tree is given to the overall model, tree complexity refers to the number of nodes per tree and thus interactions that are modeled, and the bag fraction determines the randomly selected proportion of data to be used for each tree (Elith et al. 2008). As the learning rate is decreased, the number of trees in a BRT model increases and Elith et al. (2008) recommend developing BRT with at least 1000 trees. To determine the optimal model, investigators often assess the number of trees, cross-validated (CV) model deviance, and area under the receiver operating curve (AUC; Elith et al. 2008, Sokyan et al. 2014, Main et al. 2015). As the overall BRT model is built with a given learning rate, tree complexity, and bag fraction, the statistical software uses cross-validation to 'test' the model against a reserved portion of the dataset. In this case, at each step when the number of trees is increased, ten BRT models are built and tested against unique subsets of the data (Elith et al. 2008). This is repeated for each increase in number of trees until cross validated deviance is minimized and this becomes the optimal model, given the conditions provided, with an optimal number of trees. CV deviance represented the deviance averaged across all BRT

models as the software works through this process, whereas residual deviance is just that of the optimal model. Both may be used to assess models as better models have reduced deviance in general, but the CV is recommended because it does not use original training data to calculate the percent deviance explained (Elith et al. 2008).

Two important results from BRT models are the relative influence of each variable and the partial dependence plots (De'ath 2007, Elith et al. 2008). The relative influence of each variable is assessed by how many times the variable is selected for a tree and, when it is, how well it improves the model - typically measured by reduction in deviance (Elith et al. 2008). These values are scaled such that the relative influence values for all variables totals 100. Partial dependence plots illustrate the relationship between the response and individual explanatory variables when the effects of all other variables on the model response are held at average (De'ath 2007, Elith et al. 2008).

In this case, I used a tree complexity of 3 because of my small sample size (Elith et al. 2008) and the fact that interactions greater than three-way are difficult to explain (Main et al. 2015). I also assigned a random number between 1 and 100 to each wetland as an explanatory variable to help determine which variables are useful (i.e., better than random) in the BRT model (Soykan et al. 2014, Main et al. 2015). With this, I first repeatedly ran models with increasingly smaller learning rates from 0.005 – 0.0001 and selected the best model based on the number of trees, CV deviance, and AUC values. I then retained this learning rate value and ran three models with varying bag fractions (0.5, 0.6, and 0.7). The optimal bag fraction from this was retained for future use. At this point, I observed relative influence of the variables and dropped the random number variable and any variables with smaller influence values. Finally, I reran models with the retained tree complexity and bag fraction to find the optimal learning rate; this was the final, simplified model. I focused on the relative influence values and partial dependence plots to inform my interpretation of main effects, and, as the most important pairwise interactions between variables were not particularly insightful, I did not focus on those interactions here or discuss them further. Other ecological studies using BRT models also focus on relative influence values and partial dependence plots for interpretation (Soykan et al. 2014, Main et al. 2015). These models were built in Program R v. 3.5.1 using the *gbm* package and additional code (Elith et al. 2008, Greenwell et al. 2018).

2.3 RESULTS

2.3.1 Habitat Characteristics

Many water quality, wetland habitat, and landscape level characteristics differed across sites in 2017 and 2018 (Tables A-6, A-7, A-8). However, these differences were often not easily distinguishable between grassland (Allan, St. Denis) and cropland sites (Burr, Colonsay, Humboldt; Tables A-9, A-10, A-11). In general, wetlands were saline (TDS >133 mg/L), had basic pH (6.7 – 10.1), were well oxygenated with only 3 wetlands in each year having daytime dissolved oxygen levels below 5 mg/L, and had a wide range of turbidity levels (0.0 – 38.3 FNU), similar to other reported values of prairie wetland water chemistry variables (e.g., Rawson and Moore 1944, Hall et al. 2009). On average, every site had wetlands that would be categorized as hypereutrophic based on total nitrogen and phosphorus (Tables 2.1, 2.2, Smith et al. 1999). Based on chlorophyll a, all sites would be similarly classified as eutrophic or hypereutrophic (Tables 2.1, 2.2, Smith et al. 1999).

Of the 166 pesticides screened for in 2017 only 14 were detected at least once, and of the 172 screened for in 2018 only 16 were detected at least once, with the most commonly detected compounds in both years being the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA), and the neonicotinoid insecticide imidacloprid. Overall detection frequency was higher in 2018 with 96% of wetlands having at least one pesticide detected compared to 71% in 2017, but generally the proportion of pesticides detected and sum concentration was low at all sites in both years and, after adjusting *p*-values for multiple comparisons, there were few statistically significant differences among sites and these were in 2017 only (Tables 2.1, 2.2, A-6, A-9, A-10).

With respect to wetland habitat, sites were generally similar across all variables. Exceptions include surface area, fill code, situation, vegetation cover, and vegetation buffer width (Tables 2.3, 2.4, A-6, A-8, A-10, A-11). Differences were identified primarily at Allan where most wetlands were large and had smaller vegetation buffer zones (Tables 2.3, 2.4). The greatest source of differentiation between sites was found at the landscape level (Tables 2.5, 2.6, A-6, A-9, A-10). In general, cropland sites were composed primarily of various crop types and had less land cover composed of water and wetlands (Tables 2.5, 2.6). While the grassland sites St. Denis and Allan, did have higher proportions of water and natural land cover, St. Denis still had substantial contributions of crop land averaging around 37%, and Allan had tamed pasture.

2.3.2 Ranavirus and Wood Frog Presence/Absence

In summer 2017, ranavirus eDNA was detected at 15% of the wetlands, and this proportion differed among sites ($p = 0.014$) although pairwise post-hoc Fisher's exact tests revealed that these differences did not hold with adjusted p-values (all pairwise $p > 0.05$, Table 2.7). Detections were greatest at St. Denis where 60% of wetlands tested positive for ranavirus eDNA, and lowest at Burr, Colonsay, and Humboldt where 0% tested positive. In summer 2018, with an increased total sample size, the proportion of wetlands with ranavirus was 40%, with no difference in the proportion among sites ($p = 0.083$, Table 2.7). The greatest detection rates were at Allan and St. Denis (50%), and lowest at Burr (0%). The lack of differences among sites in both years is likely due to low sample size per site. Summer detection rate (40%) was also significantly greater than in spring (4%, $p < 0.001$, Tables 2.7, A-12).

For wood frogs, in 2017 there were positive eDNA detections at 54% of wetlands compared with 42% via traditional methods, but this difference was not statistically significant ($p = 0.579$, Table 2.8). However, in 2018, the eDNA method detected wood frogs in 69% of wetlands, significantly more than the 28% of wetlands where wood frogs were detected with traditional methods ($p < 0.001$, Fig. 2.1). Like ranavirus, detection of wood frogs using eDNA was also significantly greater in summer (69%) than in spring 2018 (35%, $p < 0.001$, Tables 2.8, A-13). In both years there was no difference among sites in the proportion of wetlands with positive eDNA wood frog detections ($p > 0.05$, Table 2.8).

2.3.3 Factors Influencing Wood Frog Presence

The simplified BRT model of wood frog presence containing only 17 variables (those that had a relative influence greater than random) generally performed better than the BRT containing all variables (Tables 2.9, A-14). After removing poorly performing variables, percent CV deviance explained increased by 3.27% and the CV receiver operating score (ROC) or AUC decreased by only 0.026 (Table 2.9). Fifteen variables performed worse than random, and after removal the most influential variables were generally the same in both models (Table A-14). Dissolved phosphorus was the most influential predictor with a relative influence score of 22.16, followed by dissolved nitrogen, proportion water, chlorophyll a, TDS, CPUE, and vegetation buffer width with scores > 5 (Fig. 2.2). In general, wood frog presence was associated with greater dissolved phosphorus, higher proportion of water on the landscape, and larger vegetation

buffer widths (Fig. 2.3a, c, g). Wood frog presence was also positively associated with approximately 0.1-0.2 mg/L dissolved nitrogen, less than about 15 μg/L chlorophyll a, 1000 mg/L total dissolved solids, 0.02 proportion of exposed land, 8.5 pH, 3 mg/L total nitrogen, 0.02 proportion of pasture, and less than 50 m from a road (Fig. 2.3b, d, e, h, i, j, k, l). Wood frog detection was also negatively associated with lower CPUE of tadpoles (Fig. 2.3f). Each of these variables differed between sites in 2018, except for dissolved nitrogen, catch-per-unit-effort, and road distance (see Tables A-6, A-7, A-10 for further detail). However, these differences were not always distinguishable between grassland and cropland sites (Fig. 2.4). All sites except Allan had high dissolved phosphorus concentrations, and grassland sites tended to have lower dissolved nitrogen concentrations, higher chlorophyll a concentrations, and greater proportions of water at the landscape level (Fig 2.4a, b, c, d); although these generalizations are not all statistically significant (Table A-10). Vegetation buffer widths varied among and within sites although buffers at Allan were the smallest (Fig 2.4g, Table A-10) due primarily to recent flooding, further evidenced by large average wetland surface area (Tables 2.1, 2.2), rather than intrusive plowing. Allan also had the greatest proportion of land use as pasture (Fig. 2.4k). Other explanatory variables showed considerable within- and among-site variation (Fig. 2.4).

2.4 DISCUSSION

This work provides insight on wood frog presence and the factors that influence it in the PPR region of Saskatchewan, Canada. I compared traditional and new (eDNA) methods for detecting wood frogs and used novel modeling methods (BRT) to evaluate the influence of multiple environmental variables at different spatial scales on wood frog presence. Generally speaking, eDNA was more successful at detecting wood frogs although there was seasonal variation in success. More importantly, however, wood frog detection did not differ between grassland or cropland sites, confirming that wood frogs are still present in these disturbed landscapes and indicating that wetland-specific characteristics are more likely to influence whether or not a wetland is utilized. The BRT showed that 17 of 32 variables were better than random in terms of their influence on wood frog presence. Variables with influence values greater than 5 were primarily associated with wetland productivity, vegetation buffer width, and the proportion of the surrounding landscape that is also water.

2.4.1 Wood frog detection and eDNA

The overall detection frequencies of wood frog presence in this study are comparable to other reports of percent detections for other anurans (9% - 83%; Mackenzie et al. 2002, Mazerolle et al. 2005, Scherer et al. 2012). In both years, eDNA was more successful than traditional survey methods at detecting wood frogs in wetlands, but this was only statistically significant in 2018 and could be for several reasons. First, it could be a matter of sample size, such that in 2017 the sample set was not large enough to elicit a statistically significant difference. It could also be a factor of time spent surveying or present working at each wetland. In 2018 the number of wetlands surveyed was almost three times greater so there was much less time spent at each wetland compared to 2017 and thus less time to make opportunistic observations of wood frogs. Regardless of the extent to which eDNA out-performed traditional survey methods, it did so in both years. Given that amphibian surveys can be challenging, eDNA is a useful method to improve our understanding of target species occurrence (Gibbs et al. 2005, Muths et al. 2005, Goldberg et al. 2018). While eDNA is becoming a well-established method for detecting cryptic or otherwise hard-to-survey species it is not always perfect (Goldberg et al. 2018), and results from this study illustrate a few limitations of this method.

The eDNA results from this study showed considerable differences in their detection of both wood frogs and ranavirus in 2018 between spring and summer sampling efforts, with detection being much greater in the summer for both targets. This is not an unfamiliar phenomenon and points to the importance of matching eDNA sampling with the ecology of the target species (Buxton et al. 2018, Goldberg et al. 2018). Although wood frogs are known to be one of the earliest anurans to breed each spring (Martof and Humphries 1959, Redmer and Trauth 2005), the exact start to the breeding season may be wetland-specific. I began collecting eDNA samples from all wetlands in the spring of 2018 (May) when I knew that breeding had begun locally, but it is possible that breeding had yet to begin at some study wetlands by the time I collected eDNA samples, evidenced by negative eDNA detections even though I collected tadpoles in the summer. Since high pH and low average temperatures in the wetlands meant that eDNA degradation rates were likely low, other discrepancies between traditional and eDNA detection may be due to too few water samples collected from each wetland, microbial degradation, or PCR inhibition (Goldberg et al. 2016, Goldberg et al. 2018). The influence of wood frog abundance on eDNA detection is illustrated in the BRT model as well, such that very

low CPUE values (< 0.01) had a negative influence on wood frog detection. Based on these results, using eDNA detection as a survey method for wood frogs, and likely other pond-breeding amphibians, may be more effective when conducted in the summer after surveyors can be certain that breeding has commenced. With regard to the seasonal detection of ranavirus, this may be similarly accounted for by the ecology of wood frogs whereby sublethally infected adults returning to ponds to breed or otherwise utilize a wetland after overwintering act as intraspecific reservoirs and reintroduce ranavirus (Brunner et al. 2004, Brunner et al. 2015). Ranavirus may also be introduced to wetlands by other aquatic species (Schock et al. 2008, Miller et al. 2011, Brunner et al. 2015) or by the movement of water and soil through agricultural activities (Gray et al. 2009), and may persist in a wetland over winter in frozen carcasses (Brunner et al. 2015). Of note, the assay used to detect ranavirus here can detect multiple species and strains, not all of which may be harmful to wood frogs (Picco et al. 2007, Duffus et al. 2015, Hall et al. 2016).

An important finding of the eDNA results is that the frequency of wood frog detection did not differ between grassland and cropland sites and supports other findings of wood frogs in disturbed habitats (Knutson et al. 2004, Porej et al. 2004). This suggests that wood frogs may be able to adapt to and survive in changing landscapes, but they are also subject to associated habitat changes that may have negative effects on aspects of their life history and ecology including immune system function (Mann et al. 2009), reproductive success (Regosin et al. 2002, Knutson et al. 2004), population connectivity (Trenham et al. 2003), and habitat suitability (Porej et al. 2004, Gibbs et al. 2005). Previous research suggests that wood frogs may be more resilient to habitat patchiness and connectivity, likely due to their large dispersal capability (Berven and Grudzien 1990, Marsh and Trenham 2001, Newman and Squire 2001), but individuals may still be stressed or killed while traversing agricultural landscapes (Fahrig et al. 1995, Vos et al. 2007, Swanson et al. 2018). If suitable habitat becomes sufficiently isolated in disturbed landscapes, however, population level impacts are likely to occur, especially with regards to juvenile dispersal (Marsh and Trenham 2001, Cushman 2006, Vos et al. 2007). Despite their presence in agricultural landscapes, the results from this work show that wetland-specific characteristics are indicative of wood frog presence or absence, and it is these qualities that must be the focus of conservation and management actions, especially in altered landscapes.

2.4.2 Influences on wood frog presence

Modeling wood frog presence or absence revealed that about half of measured environmental variables were more influential than random. Of those with a relative influence value greater than 5, they were primarily associated with wetland productivity (dissolved phosphorus, dissolved nitrogen, chlorophyll a), the vegetation buffer around a wetland, and the proportion of water within a 1 km radius of the center of the wetland. Wood frog presence was positively influenced when dissolved phosphorus was ≥ 0.4 mg/L, when dissolved nitrogen was between approximately 0.1 and 0.2 mg/L, and when chlorophyll a was ≤ 15 µg/L. Lake-specific total phosphorus and nitrogen, and chlorophyll a criteria suggest that all study wetlands are either eutrophic or hypereutrophic (Smith et al. 1999), but while significant algae growth was observed in some wetlands there were no other signs of eutrophic-related hypoxia observed (e.g., extremely low dissolved O_2 concentrations, dead animal life). Even when dissolved oxygen levels are fairly low, better mixed systems, like wetlands, are less likely to reach states of hypoxia (Diaz 2001) and are naturally prone to higher nutrient concentrations, especially of phosphorus (Serrano et al. 2017). Further, using lake-based criteria to assess wetland trophic status may be misleading due to differences in nutrient cycling between wetland water and sediment (Serrano et al. 2017). The nutrient and chlorophyll a concentrations found here indicate that these wetlands are naturally eutrophic and it makes sense that frogs are more likely to be present in systems with greater food availability. The threshold for dissolved phosphorus suggests that sufficient wetland productivity is needed for wood frog habitat in this cold, dry landscape, but the limit for chlorophyll a suggests a weak or negative relationship between dissolved nutrients and suspended algal biomass, instead pointing to other primary producers (e.g. periphyton, submerged and emergent vegetation) as possible users of high nutrients in these systems. Highly productive wetlands are likely to provide abundant food supplies for frog larvae and adults, and some evidence exists to suggest that eutrophication may increase tadpole growth in this way (Belden 2006, Johnson et al. 2007). None of the wetlands had dissolved nitrogen (nitrate) levels similar to those that can cause tadpole mortality or sublethal effects (Marco et al. 1999, Camargo et al. 2005, Krishnamurthy et al. 2008, Smith et al. 2011). All wetland nitrate concentrations observed in this study were also below the recommended maximum value of 2.0 mg/L NO_3-N for sensitive freshwater species (Camargo et al. 2005).

Vegetation surrounding wetlands provides important habitat for amphibians for foraging, overwintering, and for dispersing juveniles (Semlitsch 2002, Semlitsch and Bodie 2003, Swanson et al. 2018). Vegetation buffers in disturbed landscapes, specifically, may also provide refuge from terrestrial contaminant exposure (Swanson et al. 2018), and reduce exposure to contaminants entering wetlands through run-off (Reins et al. 2013, Main et al. 2015) or aerial spray (Thompson et al. 2004). Here, I found that wetlands with vegetation buffers less than approximately 10 m in width were less likely to have wood frogs. This supports other research demonstrating positive effects of vegetation buffers on amphibian occurrence for individual species and for amphibian communities. Stapanian et al. (2015) found that wetland-level habitat alteration was a strong predictor of several amphibian response variables, including the presence of wood frogs. Measures of habitat alteration were also good predictors of wetland vegetation quality indicating that unaltered or minimally altered wetlands maintain healthy vegetative and amphibian communities (Stapanian et al. 2015). The way that I measured vegetation buffer width was also an indirect measure of wetland disturbance, i.e., narrower vegetative buffers were generally associated with plowing or flooding. Swanson et al. (2018) found that grassland buffers were frequently occupied by northern leopard frogs and had the lowest exposure concentrations of pesticides. Vegetation buffers are clearly important for both larval and adult frogs at the wetland scale, but landscape level factors are also important for wood frog presence.

Landscape level variables are frequently implicated for their effects on amphibian occupancy of wetland habitats because habitat loss and destruction are one of the greatest threats to amphibian declines (Cushman 2006, Hopkins 2007, Mann et al. 2009). Previous research has found that proximity and abundance of forested habitat to wetlands is positively associated with amphibian diversity and individual species presence and persistence, including the wood frog (Guerry and Hunter 2002, Houlahan and Findlay 2003, Porej et al. 2004). However, many studies looking at landscape level impacts on amphibians are conducted in regions where forests were naturally present but have since been degraded or destroyed including Maine (Guerry and Hunter 2002), Ontario (Houlahan and Findlay 2003), and Ohio (Porej et al. 2004). In contrast, this study was conducted in the PPR where forested landcover is naturally less common, thus making it a unique region of the wood frog's vast range. The results here indicate that more water and wetlands at the landscape level (within a 1 km radius from the wetland center) is positively associated with wood frog presence rather than forest cover. Several others have also

noted that number of wetlands and proximity to wetlands are good predictors of amphibian presence in wetland habitats (Houlahan and Findlay 2003, Trenham et al. 2003) and may improve metapopulation dynamics in an altered landscape (Marsh and Trenham 2001, Cushman 2006). However, the proportion of water at the landscape level was also positively associated with the proportion of natural habitat (including forests, $r = 0.71$) and negatively correlated with proportion crops ($r = -0.94$). Larger wetlands and those with substantial buffers (e.g., willows and other shrubs) are presumably more difficult to drain and consolidate, and this may explain at least some of the correlation between water and natural land cover. It is clear though that as the proportion of crop land increases, the proportion of water decreases. Much of the natural prairie landscape in central Canada has been converted to crop land and wetlands are increasingly being drained and consolidated to increase total area available for crops (Huel 2000). Reducing wetland loss would help wood frogs persist in these agricultural landscapes through several mechanisms, such as (1) retaining more aquatic habitat and indirectly preserving some natural habitat, and (2) likely improving or supporting wood frog metapopulation dynamics. Finally, preserving wetlands for wood frogs would inherently preserve habitat for many other taxa in these wetland communities as well.

Two other variables that had > 5 relative influence scores were TDS and CPUE (for discussion on CPUE see Section 2.4.1). TDS measures the concentration of many ions but without specific knowledge of the ionic composition in question it is difficult to draw conclusions. The BRT indicates that TDS <1000 mg/L was positively associated with wood frog presence and that above this value there was a slightly negative association, although wood frogs were occasionally detected in wetlands with TDS >2000 mg/L. Some research has been done on the toxicity of TDS to freshwater aquatic organisms, mostly with standard test organisms, but Chapman et al. (2000) did find reduced growth and survival in chironomid larvae at TDS levels around 2000 mg/L. With regards to salts and anurans, much work has focused on road deicing salts. Collins and Russell (2009) found that wetlands with high chloride concentrations had fewer amphibian species, including the wood frog. Research has identified toxicity thresholds of chloride for tadpoles, but there is also evidence for local population adaptation to saline environments (Collins and Russell 2009, Hopkins and Brodie 2015). While the potential for road salts to be toxic to amphibians is an important topic, road salts and chloride are not a concern at these study sites because salt is not used for de-icing. Instead, many wetlands are

naturally high in sulfate and magnesium ions due to soil and groundwater inputs and a lack of surface flow outlets (Rawson and Moore 1944).

The results of this modeling effort indicate that habitat variables at all levels – water quality, wetland habitat, and land use – have an influence on wood frog presence in the PPR. However, in using eDNA as a way to assess wood frog presence or absence there are a few caveats. One, eDNA may lead us to conclude that wood frogs were not present at a site when they actually were, and thus commit Type II error (false negative). This may happen if the water sampling protocol is not sufficient given wetland size, if wood frog eDNA in a given wetland is scarce or not well distributed, or if a wood frog has previously been at a wetland but its DNA has since degraded (Goldberg et al. 2016). Second, just because a wood frog is present at a wetland based on eDNA detection, it does not mean that the wetland itself is good habitat for breeding and reproductive success or that the wetland supports a healthy wood frog population (Goldberg et al. 2016). Finally, the modeling approach used here may also have benefitted from increased sample size to improve model metrics (deviance explained, AUC) and the inclusion of additional explanatory variables such as winter snowfall (Donald et al. 2011), vegetation species composition (Brogan and Relyea 2015), presence or abundance of pathogen host snail species (Rohr et al. 2008), and the inclusion of glyphosate in the pesticide screenings which was not done here due to analytical constraints. This study contributes to the pre-existing body of knowledge investigating the effects of environmental variables on frog presence, but also serves as a foundation for future investigations into wood frog presence specifically in the PPR and may be built upon by increasing the number and geographical scope of surveyed wetlands.

2.4.3 Conservation and Management Implications

Although ranavirus was not identified by the BRT model as an important factor in predicting wood frog presence or absence in the PPR, its importance overall should not be dismissed. The lack of influence of ranavirus presence on wood frog presence may simply be because die-offs of tadpoles occurring in one year does not eliminate adult frog presence at the same wetland in the following year (e.g., see Hall et al. 2018). The PPR is a relative hotspot of amphibian diversity in Canada (Lesbarrères et al. 2014) and monitoring plans for emerging infectious diseases of amphibians should be considered (Lesbarrères et al. 2012). Despite some reports of ranavirus-related die-offs in Saskatchewan (Bollinger et al. 1999, Schock et al. 2008) it is possible for amphibian species to persist in habitats with ranavirus, but future changes in

virulence or environmental factors may affect the degree to which ranavirus is a threat to PPR amphibians (Schock et al. 2008, Lesbarrères et al. 2012, Brunner et al. 2015, Hall et al. 2018). Finally, improvements in identifying ranavirus strains will also improve our understanding of disease ecology in an environment where hosts appear to persist in light of disease presence (Lesbarrères et al. 2012, Brunner et al. 2015).

Pesticide detections and sum concentrations were also insufficient at predicting wood frog presence or absence despite the risk of overestimating the effect of pesticides with the use of 'sum concentration.' It is possible that frogs may sense pesticides to avoid them (Takahashi 2007), but the lack of a pesticide effect here indicates that wood frogs in this region are not associated with wetlands that have high or low pesticide presence or total concentration. This is true even at the sites identified *a priori* as grassland locations where pesticides were still detected in wetlands. This is likely due to spray drift from nearby agricultural activities, but even at the St. Denis National Wildlife Area there is prescribed use of herbicides to control alien plant species (EC 2013). Apparently undeterred by pesticides, wood frogs are clearly at risk of chronic exposure to pesticide and fertilizer mixtures, especially in the larval stage, that may have additive, synergistic, or antagonistic interactions (Mann et al. 2009). However, there is little evidence for negative effects of the most commonly detected pesticides here, namely 2,4-D, MCPA, and imidacloprid (Relyea 2005, Johnasson et al. 2006, Robinson et al. 2017, Lee-Jenkins and Robinson 2018). Without further investigation into the effects of chronic exposure to agricultural contaminants on wood frog tadpoles it is difficult to say if there is potential for population level effects, but this work illustrates the need for field-based investigations of toxicity.

Perhaps the most important result of this study is that while the relative importance of productivity, vegetation buffers, and proportion water variables do differ, they are all influential in determining wood frog presence in the PPR. This has complex consequences for management decisions because each of these factors must be kept in mind if the goal is to provide suitable wood frog habitat in an agriculturally altered landscape. Currently there is limited management in Saskatchewan with regards to preventing continued wetland loss and consolidation (Pankratz 2010). Preserving wetlands on the landscape is a must to conserve the wildlife communities and species that rely on wetlands for part or all of their life cycles, but at the same time we must also be aware of water quality and preserving wetland habitat (e.g., vegetation buffers). The results

of this work indicate that wood frogs may persist in a dramatically altered landscape as long as there is suitable habitat, but without maintaining and protecting critical habitat components at multiple spatial scales it is possible for habitat suitability of wetlands in an imperiled landscape to continue to decline which may have population level consequences.

Table 2.1. Mean, median, standard deviation, minimum, and maximum values of water quality variables for each site in 2017.

		DO (mg/L)	pH[b]	TDS (mg/L)[b]	Turbidity (FNU)	Chl.a (µg/L)[b]	DN (mg/L)	DP (mg/L)	Ammonia (mg/L)[a]	TN (mg/L)	TP (mg/L)	PestDet (µg/L)[a]	Pest SumConc (µg/L)[a]
Allan	Mean	7.9	8.3	2228	4.7	20.6	0.15	0.23	0.42	3.00	0.33	0.002	0.002
	Median	7.7	8.3	2332	3.9	19.5	0.08	0.21	0.43	2.93	0.28	0.000	0.000
	SD	1.0	0.2	1261	3.0	14.4	0.18	0.11	0.31	0.18	0.15	0.003	0.005
	Min	7.0	8.0	687	2.2	5.3	0.03	0.15	0.10	2.87	0.20	0.000	0.000
	Max	9.3	8.5	3561	8.9	38.2	0.41	0.38	0.70	3.26	0.55	0.006	0.009
St. Denis	Mean	8.6	8.0	1707	6.9	14.2	0.05	0.43	0.88	4.02	0.41	0.004	0.043
	Median	9.6	7.8	2115	6.3	16.1	0.04	0.33	0.87	3.73	0.40	0.000	0.000
	SD	6.4	0.6	1117	2.1	10.2	0.02	0.20	0.14	1.27	0.20	0.005	0.094
	Min	1.0	7.5	542	3.9	3.8	0.03	0.26	0.72	2.67	0.22	0.000	0.000
	Max	16.1	8.8	3073	9.0	27.2	0.09	0.76	1.10	6.11	0.72	0.012	0.211
Burr	Mean	11.4	8.9	517	12.6	39.3	0.08	0.59	0.17	3.72	0.62	0.010	0.098
	Median	11.2	8.8	516	13.2	21.8	0.08	0.46	0.09	3.61	0.58	0.012	0.077
	SD	1.5	0.3	84	10.3	41.2	0.03	0.42	0.16	0.28	0.20	0.007	0.092
	Min	9.6	8.6	394	1.8	4.6	0.05	0.22	0.04	3.49	0.36	0.000	0.000
	Max	13.6	9.5	620	23.5	101.2	0.12	1.25	0.43	4.25	0.87	0.018	0.248
Colonsay	Mean	8.9	8.6	1032	5.2	6.9	0.10	0.62	0.77	3.81	0.61	0.024	0.877
	Median	8.8	8.7	894	4.2	2.4	0.09	0.52	0.51	3.31	0.53	0.024	0.719
	SD	0.3	0.6	373	3.8	9.6	0.07	0.28	0.68	1.79	0.23	0.015	0.551
	Min	8.6	7.9	623	1.5	1.1	0.01	0.43	0.29	2.42	0.46	0.006	0.295
	Max	9.5	9.4	1617	11.1	25.8	0.21	1.16	2.10	7.34	1.07	0.042	1.786
Humboldt	Mean	10.0	8.8	1496	7.2	2.9	0.04	0.60	0.64	3.43	0.57	0.014	0.561
	Median	11.0	8.8	1343	6.6	2.2	0.04	0.58	0.66	3.45	0.54	0.012	0.378
	SD	1.7	0.4	431	3.9	1.6	0.01	0.32	0.22	0.46	0.31	0.009	0.455
	Min	7.1	8.2	1076	1.4	1.4	0.03	0.12	0.32	2.98	0.12	0.006	0.165
	Max	11.1	9.3	2066	11.2	5.5	0.05	0.96	0.88	4.10	0.95	0.030	1.320

[a] Significant differences between sites detected via Kruskal-Wallis tests (see Table A-6). [b] Significant differences between sites detected via ANOVA (see Table A-7).

Table 2.2. Mean, median, standard deviation, minimum, and maximum values of water quality variables for each site in 2018.

		DO (mg/L)[b]	pH[b]	TDS (mg/L)[a]	Turbidity (FNU)[a]	Chl.a (µg/L)[b]	DN (mg/L)	DP (mg/L)[a]	Ammonia (mg/L)	TN (mg/L)[a]	TP (mg/L)[a]	PestDet (µg/L)[a]	Pest SumConc (µg/L)[a]
Allan	Mean	7.1	8.3	1871	7.8	21.6	0.18	0.12	0.67	2.54	0.27	0.006	0.051
	Median	7.8	8.5	1864	7.4	13.0	0.11	0.08	0.63	2.91	0.18	0.006	0.027
	SD	2.6	0.4	1172	3.1	18.3	0.16	0.13	0.50	1.01	0.30	0.000	0.047
	Min	2.2	7.8	542	3.6	6.4	0.06	0.04	0.11	0.61	0.05	0.006	0.027
	Max	9.5	8.7	3456	12.9	45.4	0.49	0.40	1.30	3.37	0.79	0.006	0.122
St. Denis	Mean	7.9	8.0	888	2.7	21.1	0.17	0.57	0.49	3.37	0.68	0.006	0.035
	Median	8.5	8.0	885	1.7	7.5	0.09	0.46	0.52	3.45	0.57	0.006	0.033
	SD	2.8	0.6	485	2.9	28.8	0.18	0.35	0.25	0.60	0.43	0.004	0.026
	Min	2.7	6.7	133	0.0	3.2	0.05	0.17	0.11	2.40	0.18	0.000	0.000
	Max	11.1	9.4	1799	9.0	94.2	0.65	1.09	0.80	4.63	1.65	0.012	0.074
Burr	Mean	10.2	8.5	441	2.2	10.2	0.19	0.68	0.42	2.36	0.86	0.026	0.288
	Median	10.1	8.5	431	0.4	5.1	0.13	0.64	0.39	2.36	0.79	0.023	0.144
	SD	2.3	0.3	84	3.1	11.5	0.15	0.38	0.38	0.23	0.53	0.010	0.348
	Min	6.5	8.2	293	0.0	1.3	0.05	0.02	0.08	1.92	0.13	0.017	0.077
	Max	15.2	9.0	601	8.4	30.5	0.51	1.22	1.35	2.70	1.95	0.041	0.901
Colonsay	Mean	10.2	8.8	1179	5.7	7.6	0.22	0.64	0.45	3.74	0.81	0.024	0.319
	Median	9.9	8.7	1141	2.3	2.0	0.15	0.61	0.48	3.55	0.70	0.029	0.250
	SD	2.6	0.5	312	11.5	16.4	0.15	0.34	0.29	1.10	0.42	0.013	0.181
	Min	5.8	8.1	782	0.7	0.9	0.07	0.32	0.00	2.68	0.34	0.006	0.194
	Max	14.1	9.8	1648	38.3	54.2	0.49	1.29	0.85	6.13	1.65	0.035	0.629
Humboldt	Mean	12.3	9.4	1516	1.4	5.8	0.34	0.33	0.81	3.23	0.43	0.029	1.975
	Median	12.2	9.4	1309	0.5	4.4	0.30	0.28	0.58	2.86	0.41	0.026	0.520
	SD	2.8	0.5	566	1.7	6.7	0.17	0.25	0.82	0.85	0.29	0.021	3.246
	Min	7.3	8.3	919	0.0	0.3	0.12	0.03	0.05	1.95	0.07	0.012	0.047
	Max	16.8	10.1	2421	4.6	24.1	0.70	0.76	2.90	4.54	0.83	0.052	6.815

[a] Significant differences between sites detected via Kruskal-Wallis tests (see Table A-6). [b] Significant differences between sites detected via ANOVA (see Table A-7).

Table 2.3. Mean, median, standard deviation, minimum, and maximum values of wetland habitat variables for all sites in 2017.

		Surface Area (m^2)	Bovid	Fish	Connectivity	Fill Code	Road Dist (m)	Situation	Classification	Veg Cover	Veg BuffWidth	Algae Cover
Allan	Mean	66031.5	0.3	0.0	0.5	4.0	3.0	4.8	4.5	3.3	2.7	6.3
	Median	73157.0	0.0	0.0	0.5	4.0	0.0	5.0	5.0	3.5	3.1	5.0
	SD	52336.2	0.5	0.0	0.6	0.0	6.0	0.5	1.0	1.0	2.0	7.5
	Min	3021.0	0.0	0.0	0.0	4.0	0.0	4.0	3.0	2.0	0.0	0.0
	Max	114791.0	1.0	0.0	1.0	4.0	12.0	5.0	5.0	4.0	4.8	15.0
St. Denis	Mean	13643.0	0.2	0.0	0.2	3.0	55.8	3.8	4.4	2.6	7.1	17.0
	Median	4459.0	0.0	0.0	0.0	3.0	70.0	4.0	5.0	3.0	5.7	10.0
	SD	17979.0	0.4	0.0	0.4	1.2	52.9	1.6	0.9	0.9	5.1	17.2
	Min	788.0	0.0	0.0	0.0	1.0	0.0	1.0	3.0	1.0	2.1	0.0
	Max	43097.0	1.0	0.0	1.0	4.0	106.0	5.0	5.0	3.0	14.8	40.0
Burr	Mean	10912.8	0.0	0.7	0.0	3.5	31.2	4.0	4.8	3.2	10.8	5.3
	Median	9179.5	0.0	1.0	0.0	3.5	0.0	4.0	5.0	3.0	13.3	6.0
	SD	7392.9	0.0	0.5	0.0	0.5	48.5	0.0	0.4	0.4	5.7	4.5
	Min	1522.0	0.0	0.0	0.0	3.0	0.0	4.0	4.0	3.0	3.5	0.0
	Max	23525.0	0.0	1.0	0.0	4.0	101.0	4.0	5.0	4.0	15.9	10.0
Colonsay	Mean	10504.8	0.0	0.0	0.2	3.8	4.8	3.8	4.7	3.0	4.6	10.8
	Median	4339.5	0.0	0.0	0.0	4.0	0.0	4.0	5.0	3.0	5.5	5.0
	SD	12751.7	0.0	0.0	0.4	0.4	8.0	1.0	0.8	0.0	2.0	14.6
	Min	869.0	0.0	0.0	0.0	3.0	0.0	2.0	3.0	3.0	1.6	0.0
	Max	31946.0	0.0	0.0	1.0	4.0	19.0	5.0	5.0	3.0	6.3	40.0
Humboldt	Mean	4748.8	0.0	0.0	0.6	3.8	72.2	4.6	4.8	2.8	7.5	7.0
	Median	2566.0	0.0	0.0	1.0	4.0	86.0	5.0	5.0	3.0	6.2	5.0
	SD	5291.8	0.0	0.0	0.5	0.4	43.0	0.5	0.4	0.4	5.4	5.7
	Min	1263.0	0.0	0.0	0.0	3.0	0.0	4.0	4.0	2.0	2.4	0.0
	Max	14125.0	0.0	0.0	1.0	4.0	113.0	5.0	5.0	3.0	16.2	15.0

Table 2.4. Mean, median, standard deviation, minimum, and maximum values of wetland habitat variables for all sites in 2018.

		Surface Area (m^2)[b]	Bovid	Fish	Connectivity	Fill Code[c]	Road Dist (m)	Situation[c]	Classification	Veg Cover[c]	Veg BuffWidth[a]	Algae Cover
Allan	Mean	60607.3	0.3	0.0	0.7	4.0	46.7	3.7	4.8	3.8	1.2	11.7
	Median	71186.5	0.0	0.0	1.0	4.0	0.0	4.0	5.0	4.0	1.5	0.0
	SD	51786.5	0.5	0.0	0.5	0.0	108.5	0.8	0.4	0.4	1.0	28.6
	Min	571.0	0.0	0.0	0.0	4.0	0.0	2.0	4.0	3.0	0.0	0.0
	Max	114791.0	1.0	0.0	1.0	4.0	268.0	4.0	5.0	4.0	2.3	70.0
St. Denis	Mean	7372.9	0.2	0.0	0.2	2.9	72.9	2.8	3.9	2.3	11.1	18.5
	Median	1055.5	0.0	0.0	0.0	3.0	58.5	3.0	3.5	3.0	10.9	0.0
	SD	13733.4	0.4	0.0	0.4	1.2	73.6	1.7	1.0	1.2	5.1	35.4
	Min	167.0	0.0	0.0	0.0	0.0	0.0	1.0	3.0	1.0	3.3	0.0
	Max	43097.0	1.0	0.0	1.0	4.0	233.0	5.0	5.0	4.0	21.4	90.0
Burr	Mean	8273.7	0.0	0.4	0.4	3.1	89.0	3.7	3.3	1.9	20.0	21.1
	Median	7993.0	0.0	0.0	0.0	3.0	86.0	4.0	3.0	1.0	23.7	0.0
	SD	7073.6	0.0	0.5	0.5	0.8	95.0	1.3	1.7	1.1	11.7	34.4
	Min	1522.0	0.0	0.0	0.0	2.0	0.0	1.0	0.0	1.0	5.6	0.0
	Max	23525.0	0.0	1.0	1.0	4.0	212.0	5.0	5.0	3.0	35.8	100.0
Colonsay	Mean	9234.4	0.0	0.0	0.2	3.9	38.4	4.0	4.6	2.9	13.2	11.5
	Median	5400.0	0.0	0.0	0.0	4.0	0.0	4.0	5.0	3.0	10.5	12.5
	SD	9658.2	0.0	0.0	0.4	0.3	77.9	0.0	0.7	0.3	8.0	10.3
	Min	1219.0	0.0	0.0	0.0	3.0	0.0	4.0	3.0	2.0	6.6	0.0
	Max	31946.0	0.0	0.0	1.0	4.0	232.0	4.0	5.0	3.0	28.3	30.0
Humboldt	Mean	3397.7	0.0	0.0	0.2	3.6	106.2	4.0	4.3	3.0	9.5	12.5
	Median	2533.0	0.0	0.0	0.0	4.0	92.0	4.0	4.5	3.0	9.5	0.0
	SD	3882.5	0.0	0.0	0.4	0.5	58.1	1.1	0.8	0.9	3.5	22.8
	Min	761.0	0.0	0.0	0.0	3.0	0.0	2.0	3.0	1.0	2.9	0.0
	Max	14125.0	0.0	0.0	1.0	4.0	206.0	5.0	5.0	4.0	16.0	60.0

[a] Significant differences between sites detected via Kruskal-Wallis tests (see Table A-6). [b] Significant differences between sites detected via ANOVA (see Table A-7). [c] Significant differences between sites detected via Fisher's Exact tests (see Table A-8).

Table 2.5. Mean, median, standard deviation, minimum, and maximum values of land use variables for all sites in 2017 using land use and cover data from 2016 (Table A-1).

		PropWater[a]	PropExposed[a]	PropUrban	PropNatural[a]	PropPasture[a]	PropCrops[a]
Allan	Mean	0.326	0.017	0.040	0.119	0.464	0.034
	Median	0.332	0.018	0.039	0.114	0.459	0.030
	SD	0.022	0.001	0.007	0.014	0.032	0.009
	Min	0.296	0.015	0.032	0.109	0.431	0.027
	Max	0.345	0.018	0.048	0.140	0.507	0.047
St. Denis	Mean	0.303	0.007	0.030	0.113	0.174	0.373
	Median	0.318	0.006	0.031	0.122	0.165	0.379
	SD	0.033	0.002	0.001	0.029	0.035	0.026
	Min	0.261	0.005	0.029	0.080	0.142	0.328
	Max	0.334	0.011	0.031	0.147	0.233	0.391
Burr	Mean	0.021	0.004	0.043	0.010	0.006	0.917
	Median	0.021	0.003	0.041	0.010	0.007	0.925
	SD	0.005	0.004	0.015	0.006	0.005	0.033
	Min	0.015	0.000	0.028	0.002	0.000	0.876
	Max	0.027	0.009	0.060	0.017	0.011	0.950
Colonsay	Mean	0.081	0.016	0.046	0.016	0.013	0.827
	Median	0.084	0.018	0.046	0.017	0.015	0.825
	SD	0.013	0.005	0.011	0.003	0.012	0.023
	Min	0.058	0.008	0.029	0.012	0.000	0.804
	Max	0.093	0.020	0.058	0.020	0.025	0.864
Humboldt	Mean	0.197	0.072	0.044	0.013	0.005	0.669
	Median	0.172	0.082	0.031	0.013	0.006	0.688
	SD	0.087	0.034	0.019	0.003	0.005	0.127
	Min	0.112	0.019	0.031	0.009	0.000	0.510
	Max	0.315	0.106	0.070	0.018	0.010	0.802

[a] Significant differences between sites detected via Kruskal-Wallis tests (see Table A-6).

Table 2.6. Mean, median, standard deviation, minimum, and maximum values of land use variables for all sites in 2018 using land use and cover data from 2016 (Table A-1).

		PropWater[a]	PropExposed[a]	PropUrban[a]	PropNatural[a]	PropPasture[a]	PropCrops[a]
Allan	Mean	0.321	0.018	0.039	0.115	0.476	0.031
	Median	0.332	0.018	0.038	0.111	0.459	0.029
	SD	0.044	0.002	0.007	0.013	0.053	0.009
	Min	0.246	0.015	0.031	0.101	0.431	0.022
	Max	0.372	0.022	0.048	0.140	0.570	0.047
St. Denis	Mean	0.305	0.008	0.030	0.110	0.169	0.378
	Median	0.322	0.007	0.030	0.104	0.162	0.381
	SD	0.031	0.002	0.001	0.032	0.027	0.021
	Min	0.260	0.005	0.029	0.079	0.142	0.328
	Max	0.334	0.011	0.031	0.149	0.233	0.404
Burr	Mean	0.021	0.003	0.037	0.009	0.004	0.927
	Median	0.019	0.000	0.028	0.006	0.000	0.942
	SD	0.004	0.004	0.014	0.006	0.005	0.030
	Min	0.015	0.000	0.026	0.002	0.000	0.876
	Max	0.027	0.009	0.060	0.017	0.011	0.951
Colonsay	Mean	0.080	0.016	0.051	0.015	0.010	0.828
	Median	0.078	0.016	0.054	0.016	0.000	0.816
	SD	0.027	0.007	0.009	0.003	0.013	0.032
	Min	0.040	0.004	0.033	0.011	0.000	0.791
	Max	0.133	0.026	0.060	0.020	0.025	0.884
Humboldt	Mean	0.161	0.061	0.041	0.012	0.004	0.722
	Median	0.119	0.055	0.031	0.011	0.004	0.789
	SD	0.093	0.043	0.017	0.003	0.004	0.146
	Min	0.060	0.011	0.030	0.009	0.000	0.499
	Max	0.315	0.138	0.070	0.018	0.010	0.873

[a] Significant differences between sites detected via Kruskal-Wallis tests (see Table A-6).

Table 2.7. Tally of positive ranavirus detections with eDNA and non-detects in each survey season with percent in parentheses.

	Summer 2017		
	Wetlands	eDNA	Not detected
Allan	4	1 (25%)	3 (75%)
St. Denis	5	3 (60%)	2 (40%)
Burr	6	0 (0%)	6 (100%)
Colonsay	6	0 (0%)	6 (100%)
Humboldt	5	0 (0%)	5 (100%)
	Spring 2018		
Allan	6	0 (0%)	6 (100%)
St. Denis	14	0 (0%)	14 (100%)
Burr	11	1 (9%)	10 (91%)
Colonsay	18	1 (6%)	17 (94%)
Humboldt	22	1 (5%)	21 (95%)
	Summer 2018		
Allan	6	3 (50%)	3 (50%)
St. Denis	14	7 (50%)	7 (50%)
Burr	9	0 (0%)	9 (100%)
Colonsay	17	8 (47%)	9 (53%)
Humboldt	19	8 (42%)	11 (58%)

Table 2.8. Tally of positive wood frog detections with traditional survey methods only, eDNA survey methods only, both methods, and non-detects (neither method) in each survey season with percent in parentheses.

	Summer 2017				
	Wetlands	Traditional	eDNA	Both	Not detected
Allan	4	3 (75%)	0 (0%)	0 (0%)	1 (25%)
St. Denis	5	3 (60%)	4 (80%)	2 (40%)	0 (0%)
Burr	6	2 (33%)	3 (50%)	2 (33%)	3 (50%)
Colonsay	6	3 (50%)	5 (83%)	2 (33%)	0 (0%)
Humboldt	5	0 (0%)	2 (40%)	0 (0%)	3 (60%)
	Spring 2018				
Allan	6	1 (17%)	2 (33%)	1 (17%)	4 (67%)
St. Denis	14	2 (14%)	2 (14%)	1 (7%)	11 (79%)
Burr	11	3 (27%)	4 (36%)	1 (9%)	5 (45%)
Colonsay	18	5 (28%)	11 (61%)	4 (22%)	6 (33%)
Humboldt	22	0 (0%)	6 (27%)	0 (0%)	16 (73%)
	Summer 2018				
Allan	6	2 (33%)	4 (67%)	1 (17%)	1 (17%)
St. Denis	14	3 (21%)	11(79%)	3 (21%)	3 (21%)
Burr	9	4 (44%)	7 (78%)	4 (44%)	2 (22%)
Colonsay	17	8 (47%)	12 (71%)	8 (47%)	5 (29%)
Humboldt	19	1 (5%)	11 (58%)	1 (5%)	8 (42%)

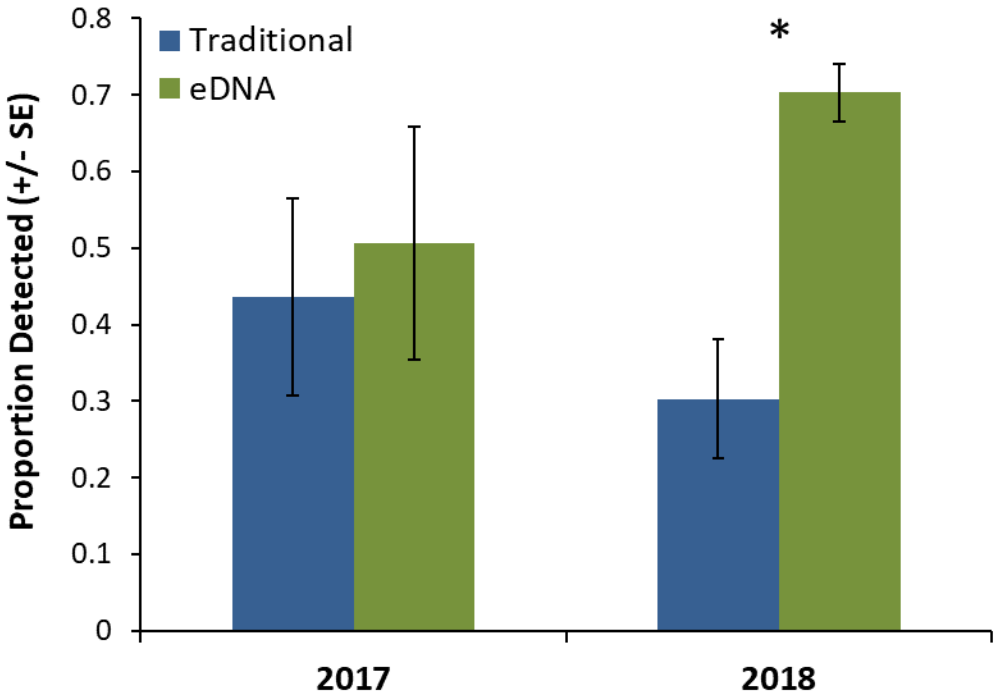

Figure 2.1. Proportion of wetlands with positive wood frog detections, averaged across sites, in 2017 and 2018, split by survey method. 26 wetlands were surveyed in 2017 and 65 in 2018. Asterisk indicates significant difference (Fisher's exact test).

Table 2.9. Parameters and evaluation metrices of the top BRT models containing all variables (WF.tc3.lr0005.bf6), and only those better than random (WF.tc3.lr0005.bf6.simp). *lr* refers to learning rate, *bf* to bag fraction, *tc* to tree complexity, *nt* to optimal number of trees, dev to deviance, and CV ROC Score translates to AUC.

Model Name	*lr*	*bf*	*tc*	*nt*	Mean Total Dev	Mean Residual Dev	Residual % Dev Explained	Estimated CV Dev	CV % Dev Explained	CV ROC Score (SE)
WF.tc3.lr0005.bf6	0.0005	0.6	3	5300	1.314	0.676	48.55	1.188	9.59	0.758 (0.071)
WF.tc3.lr0005.bf6.simp	0.0005	0.6	3	6650	1.314	0.589	55.18	1.145	12.86	0.732 (0.049)

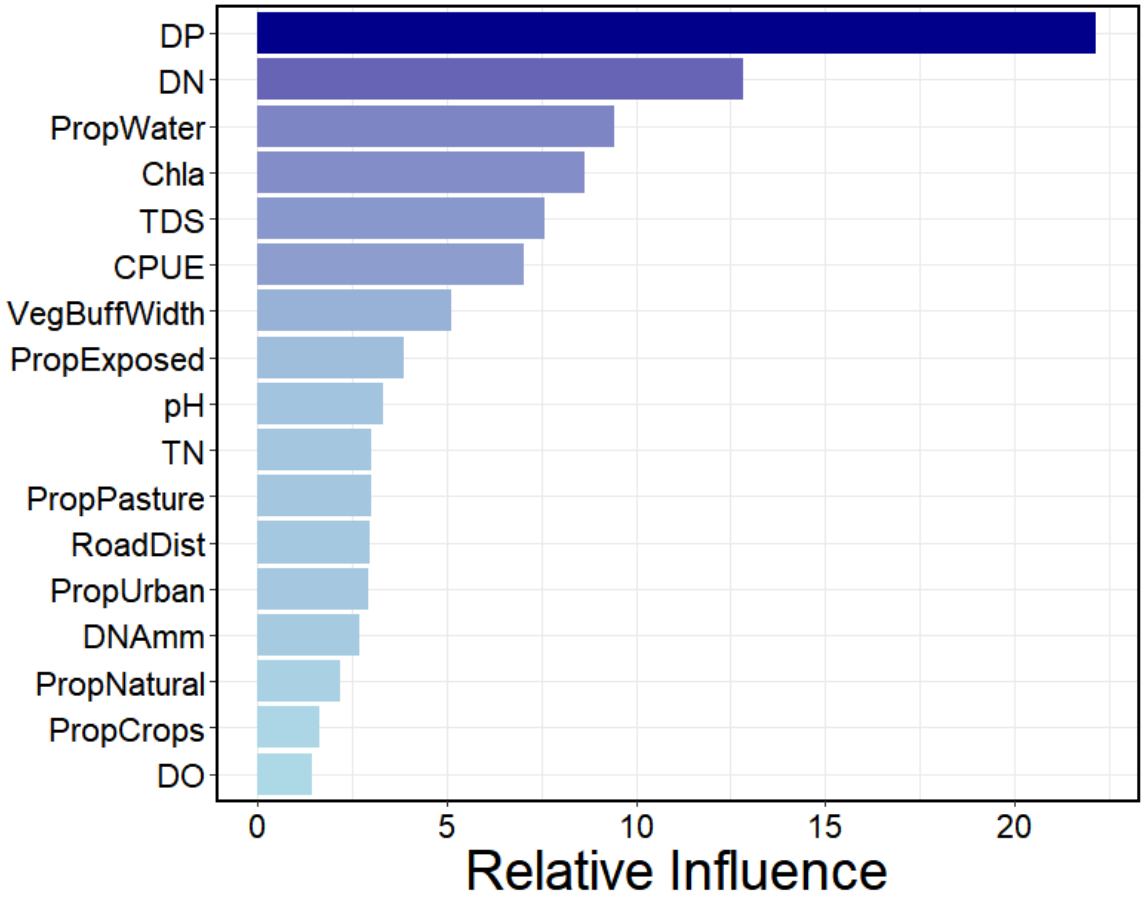

Figure 2.2. Relative influence of explanatory variables on wood frog presence after variables that performed worse than random have been removed from the BRT model. DP – dissolved phosphorus, mg/L; DN – dissolved nitrogen-nitrate, mg/L; PropWater – proportion water; Chla – chlorophyll a, µg/L; TDS – total dissolved solids, mg/L; CPUE – catch-per-unit-effort; VegBuffWidth – vegetation buffer width, m; PropExposed – proportion exposed; TN – total nitrogen, mg/L; PropPasture – proportion pasture; RoadDist – road distance, m; PropUrban – proportion urban; DNAmm – dissolved nitrogen-ammonia, mg/L; PropNatural – proportion natural; PropCrops – proportion crops; DO – dissolved oxygen, mg/L. For further detail on variables see Table A-1.

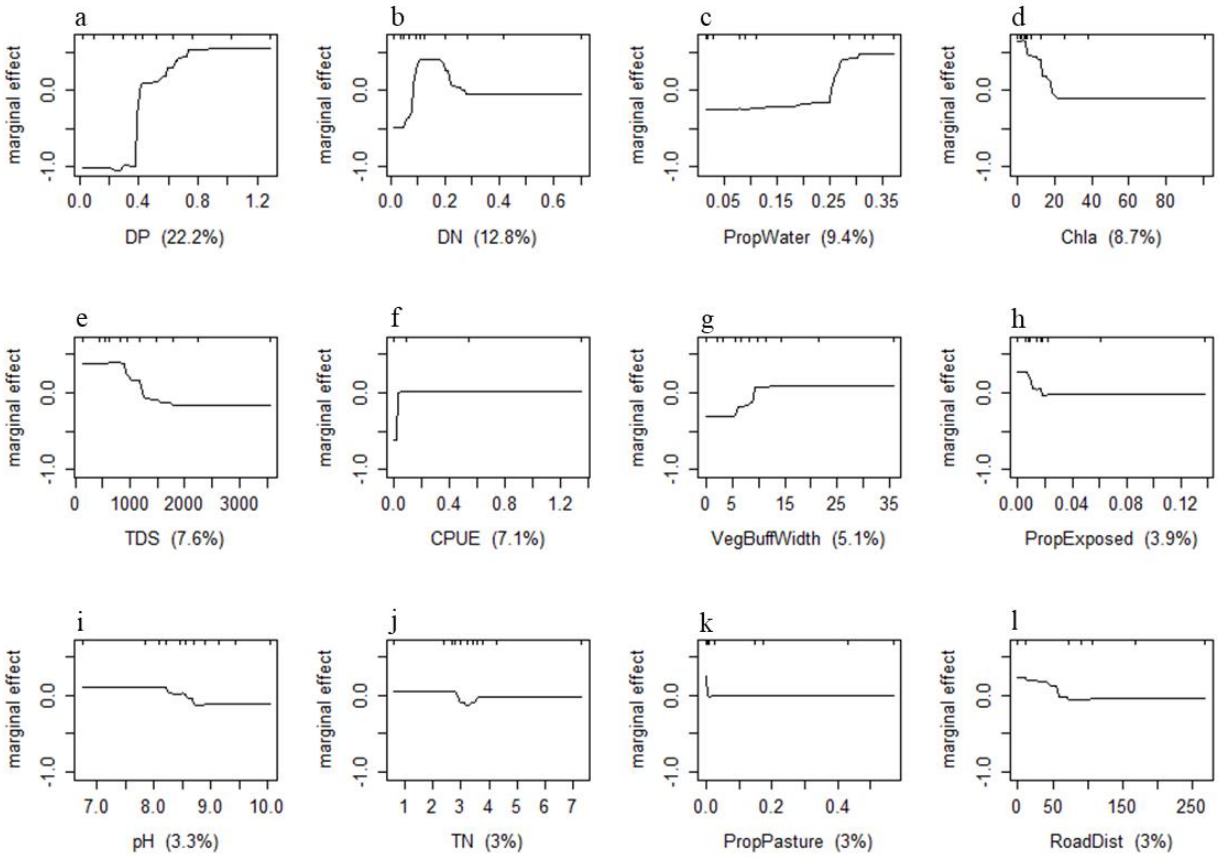

Figure 2.3. Partial dependence plots (a – l) for the 12 variables with greatest influence on wood frog presence. Marginal effect is the effect of the variable of interest on wood frog presence while the effect of the remaining variables is held at average. Percentages in parentheses are the relative influence of the respective variable. Rug plots on the top edge of each individual plot illustrate the distribution of sampling values in deciles (Elith et al. 2008). DP – dissolved phosphorus, mg/L; DN – dissolved nitrogen-nitrate, mg/L; PropWater – proportion water; Chla – chlorophyll a, µg/L; TDS – total dissolved solids, mg/L; CPUE – catch-per-unit-effort; VegBuffWidth – vegetation buffer width, m; PropExposed – proportion exposed; TN – total nitrogen, mg/L; PropPasture – proportion pasture; RoadDist – road distance, m; PropUrban – proportion urban; DNAmm – dissolved nitrogen-ammonia, mg/L; PropNatural – proportion natural; PropCrops – proportion crops; DO – dissolved oxygen, mg/L. For further detail on variables see Table A-1.

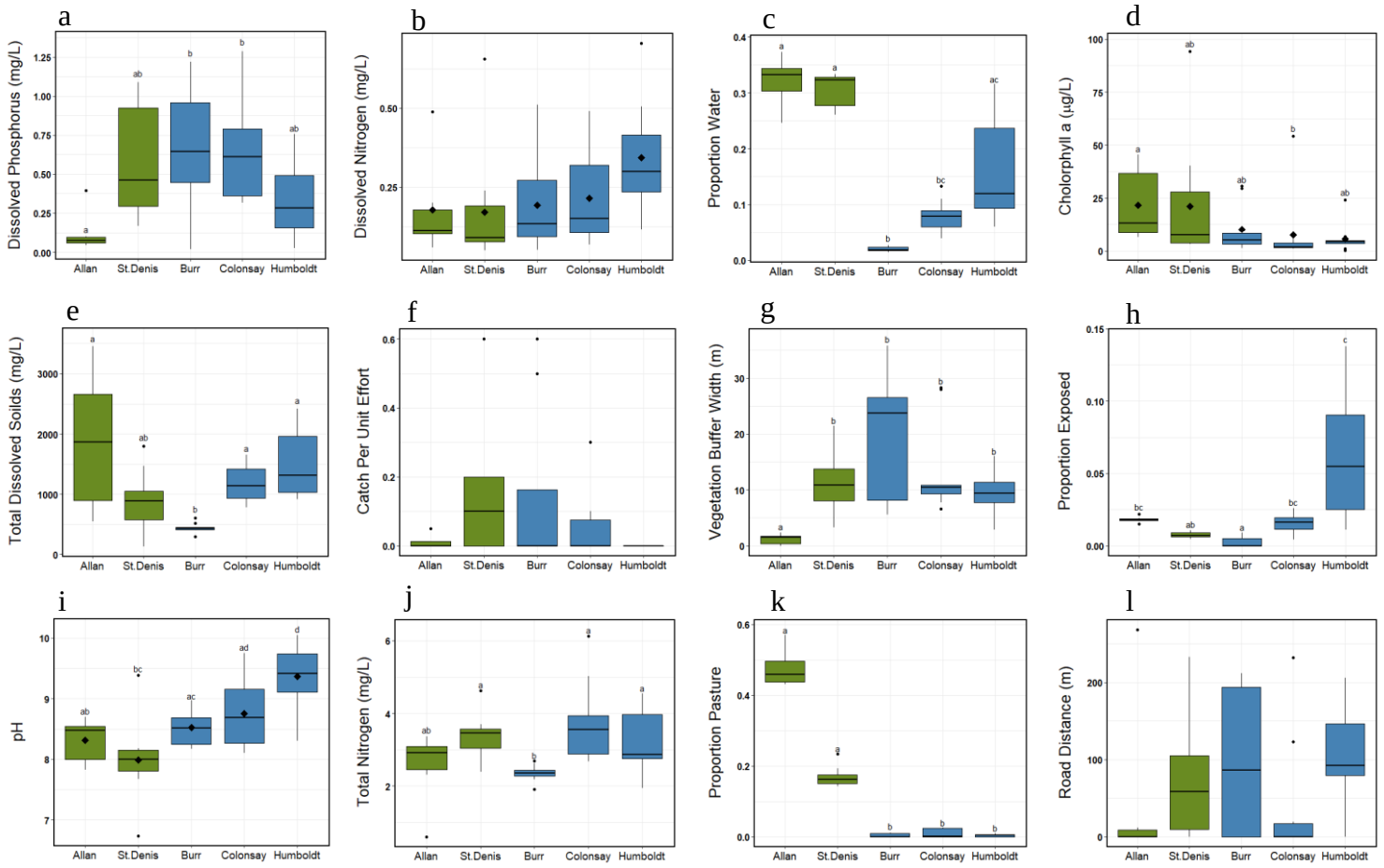

Figure 2.4. Box plots showing distribution of each variable among sites in 2018 (a – l). For variable descriptions see Table A-1. Grassland sites (Allan, St. Denis) are in green, and cropland sites (Burr, Colonsay, and Humboldt) are in blue. The bold line represents the median, edges of the box represent the 25th and 75th percentiles, whiskers reach to the furthest data point within 1.5 times the inter-quartile range, and solid dots are outliers. Black diamonds indicate mean values for normally-distributed variables. For variables in which site-specific differences were significant as determined either with Kruskal-Wallis and Dunn's tests or ANOVA and Tukey's HSD tests (see Tables A-6, A-7), shared letters indicate no significant difference.

CHAPTER 3: EFFECTS OF AGRICULTURAL LAND USE ON GROWTH AND NEUTROPHIL:LYMPHOCYTE RATIOS IN WOOD FROG (LITHOBATES SYLVATICUS) TADPOLES IN CENTRAL SASKATCHEWAN, CANADA

PREFACE

The primary aim of this study was to investigate the effects of water quality (including agricultural contaminants), wetland habitat, and land use variables on the health of wood frog tadpoles and metamorphs in Prairie Pothole wetlands of central Saskatchewan, Canada. The endpoints used to assess tadpole and metamorph health include body condition, body mass, and blood neutrophil-to-lymphocyte ratios. This chapter has a greater focus on the individual-level effects of agricultural activity on tadpoles and metamorphs. The authors and contributions of this chapter are as follows: Gabrielle E. Ruso – designed the project, collected field and lab data, analyzed the data, and drafted the manuscript; Dr. Christy A. Morrissey – provided guidance for project design and statistical analyses, managed and funded pesticide sample collection, and provided comments and edits of the manuscript; Dr. Natacha S. Hogan – provided guidance for project design, inspiration for immune system questions, and comments and edits of the manuscript; Dr. Claudia Sheedy – processed pesticide samples; Melanie J. Gallant – cultured ranavirus for positive controls; and Dr. Timothy D. Jardine – provided guidance for project design and statistical analyses, comments and edits of the manuscript, and provided research funds.

3.1 INTRODUCTION

The wood frog (*Lithobates sylvaticus*) is one of the most widespread North American amphibians with a range extending from the southeastern United States through Canada and Alaska, north of the Arctic Circle (Martof 1970, Redmer and Trauth 2005). Like most amphibians worldwide, wood frog populations have likely declined or experienced local extirpations due to habitat alterations (Redmer and Trauth 2005). As a pond-breeding anuran, the wood frog requires a variety of habitats. The species breeds in seasonal and semi-permanent wetlands free of fish, but adults may be found in a variety of habitats including tundra, woodlands and forests, and meadows (Redmer and Trauth 2005). Requiring a wide variety of

habitats throughout its life cycle may, however, put wood frogs at greater risk of detrimental effects caused by habitat loss, fragmentation, or degradation (Porej et al. 2004, Green 2005, Semlitsch and Bridges 2005).

The alteration of habitat required for just one life stage will likely have subsequent effects on the population as a whole. This is particularly true for the aquatic stage wherein tadpoles are restricted and subject to any changes in their aquatic habitat. For pond-breeding amphibians, natural processes that influence population growth rates are likely quite influential during the larval/juvenile stages and include factors such as predation, food quantity, water temperature, and the likelihood of the waterbody to desiccate prematurely (Semlitsch and Bridges 2005). The addition of anthropogenic habitat alteration puts greater stress on this life stage, in which successful metamorphosis is critical to adult survival and fecundity (Relyea 2004, Todd et al. 2011, Smith et al. 2011). A primary threat to amphibians is pollution (Hopkins 2007, Wake and Vredenberg 2008, Lesbarrères et al. 2014). Agriculture is an important source of chemical pollutants and has been identified as, perhaps, the greatest threat to Canadian herpetofauna (Lesbarrères et al. 2014). Conversion to agriculture in the Prairie Pothole Region of central Canada can cause habitat loss by destroying natural landcover and wetlands, habitat fragmentation by isolating remaining patches of natural environment, and habitat degradation by polluting and altering the vegetation of remaining wetlands.

Agricultural pollutants include fertilizers and pesticides and their effects on tadpole growth and survival are frequently studied, particularly in laboratory settings. Studies have demonstrated reduced growth and delayed development in amphibians exposed to nitrite (Griffis-Kyle 2007), and several common pesticides such as carbaryl, diazinon, malathion, and glyphosate (Relyea 2004). In general, agricultural contaminants tend to impair growth and delay metamorphosis in amphibian larvae (Mann et al. 2009), but given the complexity of contaminant mixtures, species-specific susceptibility, and environmental variation, this is not always true. Further, there is evidence to suggest that lab-based experiments may overestimate effects of contaminants compared to field studies (Lanctôt et al. 2014). Agricultural contaminants can also have indirect effects on amphibians by altering habitat and predator-prey interactions (Gibbons et al. 2015). For example, the herbicide atrazine can cause reductions in periphyton abundance and thus lead to reduced growth and delayed development in tadpoles (Rohr and Crumrine 2005). Similarly, some insecticides can indirectly lead to reduced periphyton abundance through trophic

cascades and also reduce tadpole growth and development (Relyea and Diecks 2008). Simultaneously, the broadleaf herbicide atrazine has been shown to indirectly allow for periphyton growth by reducing competition with macrophytes (Rohr et al. 2008). This promotes the growth of snail populations which, as intermediate hosts, increase trematode abundance and may lead to greater infection rates in immunosuppressed tadpoles (Rohr et al. 2008). In contrast, insecticides can cause declines in competitor abundance and thus increase periphyton availability, affecting an increase in tadpole growth and development (Rohr and Crumrine 2005). In summary, the effects of agricultural contamination on freshwater communities are complex.

Tadpole growth and development is critical for the individual's ultimate survival and fecundity as an adult (Berven and Gill 1983, Todd et al. 2011). However, in light of emerging infectious diseases, like ranavirus, it is also important to account for the effects that agricultural contaminants may have on immune system function (Mason et al. 2013), especially given the wood frog's apparently elevated susceptibility to the pathogen (Hoverman et al. 2011). One endpoint to examine for immune stress is white blood cell profiles, specifically neutrophil to lymphocyte (N:L) ratios. This ratio increases in response to elevated glucocorticoid levels, and thus may serve as a good indicator of immune system stress (Davis et al. 2008). Yet, it has been difficult to find a consistent relationship between N:L ratios and pesticide exposure, in part because of limited investigations (Shutler and Marcogliese 2011). Recent research has nevertheless illustrated that pesticide exposure can influence immune system function. Pochini and Hoverman (2017) showed that time-to-death of tadpoles challenged with ranavirus is reduced by pre-exposure to certain pesticides and that ranavirus infection can lead to a lower LC_{50} value of those pesticides.

In light of this knowledge, I investigated the potential effects of agricultural influences on tadpole size and development, and N:L ratios in a field setting. Across five sites that vary in agricultural intensity (Chapter 2), I surveyed for tadpoles and collected data on morphometrics and blood immune cell ratios. I predicted that tadpoles from the agricultural-intensive sites would show reduced growth and stressed immune systems as evidenced by elevated N:L ratios.

3.2 METHODS

3.2.1 Study Area and Wetland Selection

Tadpoles were targeted from a total of 26 wetlands at five sites between May and July of 2017 and 71 wetlands at the same five sites between May and July of 2018 as described in Chapter 2, where Allan and St. Denis represent grassland sites and Burr, Colonsay, and Humboldt represent cropland sites. In 2017, I found few wetlands containing tadpoles so in 2018 I increased the number of surveyed wetlands by establishing circular zones that contained the original wetlands surveyed in 2017. Of the 26 wetlands surveyed in 2017, 5 contained tadpoles and of the 71 surveyed in 2018, 12 contained tadpoles. At the smallest and largest wetlands within each site, I placed temperature loggers (Onset HOBO 8K pendant, UA-001-08) less than a meter below the surface of the water to record temperature once every hour and I retrieved the loggers when I completed field work. In 2017, I was able to place a pair of loggers at each site, but in 2018, I was only able to place them at St. Denis, Burr, and Colonsay. Because tadpole growth and development are likely influenced by temperature (Herreid and Kinney 1967, Smith-Gill and Berven 1979), this variable was recorded to ensure there were no large differences among sites that could confound comparisons. After retrieving data from each logger, I plotted temperature against date and time. I kept temperature values beginning from at least one hour after I placed them in the wetland to allow for acclimation. For wetlands that desiccated completely or the water level dropped below the logger before I retrieved them (2017: Burr2, Burr5, Humboldt1, Humboldt4; 2018: Colonsay5, Colonsay6, St. Denis1), I cut the last ten days of temperature data, counting back from the date and time of retrieval.

3.2.3 Tadpole and Metamorph Collection and Processing

I recorded body mass (0.1 g), snout-vent length (SVL, mm), and Gosner stage (GS; Gosner 1960) for all tadpoles collected, aiming for approximately 15 individuals per wetland. I calculated body condition following Lanctôt et al. (2014). I also collected blood samples from the base of the tail with heparinized capillary tubes for blood smears. All tissues were collected and preserved for future use at -80°C. Later in the summer I also collected 15 metamorphic frogs (GS $\geq$ 42) and processed them similarly. I again recorded mass, SVL and GS, collected blood samples for blood smears, and preserved all tissues. For metamorphs in which the tail was almost or completely absorbed, or when I could not get sufficient volume from the tail, I took

blood from the heart. If I caught more than fifteen tadpoles or metamorphs during the dipnet sweeps then the remaining individuals were released to their wetland of origin. I made blood smears in the field by smearing the blood on a microscope slide and allowing it to air dry. In the lab, I stained the slides with Protocol Hema 3 stain (Fisher Scientific Company L.L.C., Middletown, VA) and preserved them with mounted cover slips. I counted N:L ratios under a microscope at 1000x magnification by oil immersion and counted all white blood cells in a zig-zag pattern up to 100. All samples were collected under a Saskatchewan Ministry of Environment research permit (# 17FW204), an Environment and Climate Change Canada National Wildlife Area access permit (#2017-072), and with University of Saskatchewan Animal Use Protocol approval (#20170055).

3.2.5 Statistical Analyses

In order to assess the effect of individual explanatory variables related to water quality (including pesticide detection and sum concentration), wetland habitat, and land use and cover (see Chapter 2: Methods) on tadpole morphology (body condition, k, and body mass) and blood N:L ratios, I used boosted regression trees (BRT). This was selected over linear mixed effects models because BRT are more lenient towards variables of multiple scales, collinearity, and missing data (De'ath 2007, Elith et al. 2008). For each BRT model I tested the same variables as those from Chapter 2 (Table A-1), with the exception that I added Gosner stage to account for developmental effects. I did not include wetland temperatures in the models because few wetlands were monitored; rather, these data simply provide general contextual information about the wetlands. Each model also included a random number, from 1 to 100, for each tadpole or metamorph to evaluate which explanatory variables had an influence on the response variable that is greater than random. To improve normality of the response variables, I log-transformed body condition, and for N:L ratios I log-transformed by $\log_{10}(X + 0.1)$. Body mass did not require transformation. I kept tree complexity at 3 for both models, but used exploratory analyses to determine optimal values for the bag fraction and learning rate. Finally, to identify the optimal BRT models I examined percent of deviance explained. For further detail on BRT, see Section 2.2.7.2. Summary statistics were calculated in Microsoft Excel and all additional analyses were conducted in program R v. 3.5.1 (R Core Team 2018).

3.3 RESULTS

3.3.1 Wood Frog Tadpole, Metamorph, and Blood Smear Collections

In 2017, I collected tadpoles, metamorphs, and blood smears between 3 June and 21 July. In total, I collected 121 tadpoles and 7 metamorphs (Table A-15). In 2018, I collected tadpoles, metamorphs, and blood smears between 5 June and 5 July and collected a total of 125 tadpoles and 86 metamorphs (Table A-16). In both years there were cases in which the wetland desiccated before tadpoles could metamorphose, resulting in fewer metamorphs collected than tadpoles. Blood slides were collected from as many tadpoles and metamorphs as possible although there were individuals for which I could not get enough blood or it was too watery (Tables A-15, A-16). In 2017, I collected a total of 78 blood smears, and in 2018 a total of 191.

Overall mean and median tadpole body mass appeared to vary strongly across sites in 2017, with lowest mean and median values of 0.7 g and 0.6 g, respectively, at Colonsay – a cropland site, and highest values both at 2.5 g, at Burr – also a cropland site (Table A-17). Of the metamorphs collected at Colonsay, the mean and median values were 0.8 g (Table A-18). The use of mass alone, however, is misleading because of differences in the developmental stage of collected tadpoles. Tadpoles gained mass with increasing Gosner stage and SVL until metamorphosis, at which point mass declined (Fig. 3.1). The range of Gosner stages collected at Burr in 2017 was 25 – 41, whereas at Colonsay this range was 21 – 45, and at St. Denis it was 33 – 38. I visited each site at least once per week, and rotated the order of site visits to avoid consistently visiting certain sites first and others last in an attempt to collect tadpoles from each site throughout their development. However, as tadpole growth is likely influenced by temperature, my ability to collect tadpoles throughout their development was hindered by the order in which I found tadpole-containing wetlands within each site. Furthermore, the 2017 tadpoles collected at St. Denis were from a rapidly desiccating wetland. While wetland water temperatures were similar among sites (mean daily temperatures: Allan = 20.8°C, St. Denis = 19.4°C, Burr = 18.0°C, Colonsay = 20.3°C, Humboldt = 18.9°C), it is clear that wetlands of different sizes have different temperature regimes, with smaller wetlands tending towards more variable (both warmer and colder) temperatures (Fig. 3.2). Wetlands Allan3, St. Denis5, Burr5, Colonsay5, Humboldt1, and Humboldt4 were all smaller (range = 788 to 3290 m^2) and had more variable temperatures (mean coefficient of variation (CV) = 24.8) than their larger counterparts (range = 10037 to 114791 m^2, mean CV = 16.7, Fig. 3.2).

In 2018, there was less variation in mean and median mass across sites for both tadpoles and metamorphs (Tables A-17, A-18). Mean and median tadpole mass ranged from 1.5 – 2.5 g and 1.0 – 2.7 g, respectively, and mean and median metamorph mass both ranged from 1.3 – 2.1 g. The range of Gosner stages collected at each site was 34 – 44 at Allan, 31 – 46 at St. Denis, 32 – 46 at Burr, and 27 – 46 at Colonsay. Similar to 2017, I purposefully rotated site visitation throughout each week to try to capture tadpoles throughout their development, but the effectiveness of this was limited by the wetland-specific differences in temperature regimes. As in 2017, mean daily temperature did not vary widely between sites in 2018 (St. Denis = 21.5° C, Burr = 20.2° C, Colonsay = 19.9° C) but there were differences in temperature regimes according to wetland size. St. Denis1, Burr5, and Colonsay6 wetlands were generally smaller (range = 869 to 1522 m^2) than their counterparts (range = 1219 to 43097 m^2) and had more variable temperature fluctuations (mean CV = 23.9 vs 16.5, Fig. 3.3a-c).

When snout-vent-length was taken into account by calculating body condition (k), differences among sites were minimal in both years for tadpoles and metamorphs (Tables A-17, A-18). Condition was slightly lower in metamorphs (overall mean = 0.013) with site-specific averages ranging from 0.011 to 0.016, compared with tadpoles (overall mean = 0.017) with site-specific averages ranging from 0.014 to 0.022.

With regard to N:L ratios, lymphocytes were generally more common than neutrophils in both tadpoles and metamorphs, resulting in many ratio values less than 1. However, there were several cases in which neutrophil abundance was elevated such that, across all sites, N:L values ranged from 0 to 8. In both years at Burr, in particular, tadpoles and metamorphs exhibited a higher mean and a wider range of N:L ratio values (Tables A-17, A-18). This can be at least partially explained by an abundance of unique neutrophils (Fig. 3.4). Unique neutrophils were identified as neutrophils with tiny, dark pink granules in the cytoplasm. These granules tended to be very small (i.e., “pin-prick”), but appeared to occasionally clump together and look larger and irregularly shaped (Fig. 3.4). The granules were usually sparsely distributed throughout the cytoplasm, but sometimes were also fairly dense.

3.3.1 Modeling Tadpole Growth and Health

Initial runs of BRT models for body condition, N:L ratios, and mass included 32 unique variables and a random number, totaling 33 variables. The optimal BRT models for each response variable used bag fractions of 0.5 as exploratory analyses showed that increasing this

value did not improve model performance, indicated by the percent of cross-validated (CV) deviance explained (Table 3.1). Because these response variables were continuous, not binomial, I could not use a measure of area under the receiver operating curve as an indicator of model performance. The percentages of CV deviance explained by the body condition and mass BRT models were much greater than that of the N:L model (Table 3.1). For the body condition and N:L models, only Gosner stage performed better than a random number (Table 3.2). Body condition declined with increasing Gosner stage but especially so at metamorphic climax (around GS 41-42) and N:L ratio was generally stable until metamorphic climax, at which it also sharply declined.

The initial BRT model for body mass indicated that five variables had a relative influence greater than random, including Gonser stage, total dissolved solids (mg/L), proportion of pesticides detected, ammonia (NH_3-N mg/L), and wetland surface area (m^2, Table 3.2). When a simplified mass model was run, the percent of CV deviance explained increased slightly by 2.01% (Table 3.1). The partial dependence plots illustrate the influence of these five variables on tadpole and metamorph mass (Fig. 3.5). Similar to Figure 3.1, mass increased with age, i.e., Gosner stage, until metamorphic climax, and then declined (Fig. 3.5a). There is an apparent threshold for total dissolved solids of approximately 600-700 mg/L, above which there is a negative effect on mass (Fig. 3.5b). At seemingly the first sign of pesticide detection (0.01) there is also a negative influence on mass (Fig. 3.5c). With respect to ammonia however, there is generally a neutral influence on mass except between 0.5 and 0.75 mg/L where there is a small positive influence (Fig. 3.5d). Similar to ammonia, surface area has a generally neutral effect on mass except for a slight positive influence when surface area is between 4000 and 6000 m^2. Using mass as a response variable indicated that certain water quality variables are influential, but when SVL is accounted for by using body condition as the response these variables appear to be of no influence (Table 3.2).

3.4 DISCUSSION

3.4.1 General Findings

The results of this study provide evidence for successful wood frog breeding at wetlands in both grassland and cropland sites. The BRT models indicate that while there were no environmental influences on body condition or N:L ratios, growth (i.e., body mass) may be

reduced by several factors. Total dissolved solids, proportion of pesticides detected, ammonia, and surface area each influenced tadpole and metamorph mass, although only TDS and pesticides showed defined, negative effects. I also found broad differences in wetland temperature regimes, generally based on size, and observed some smaller wetlands with tadpoles desiccating before metamorphosis could be completed. In several of the desiccating wetlands, I observed tadpoles 'stress-morphing.' In response to stressors, such as pond desiccation, tadpoles may progress through metamorphosis rapidly to avoid mortality but this may have negative consequences for tadpole growth and development (e.g., mass at metamorphosis, Denver et al. 1998, Gomez-Mestre et al. 2013) and immune system function (Gervasi and Foufopoulos 2008). While wood frogs are thought of as highly philopatric (Berven and Grudzien 1990), snowfall and precipitation may influence the frequency with which adults return to wetlands to breed (Donald et al. 2011). Repetitive recruitment failure, either as failure to metamorphose or failure to breed, could result in local population declines or extinctions if conditions are sufficiently severe (Donald et al. 2011). Predicted effects of climate change in the Prairie Pothole Region include warmer temperatures and changes in precipitation regimes with the potential for more severe drought, both of which may increase desiccation of breeding wetlands and could further contribute to localized amphibian declines (Price and Waddington 2000, Winter 2000, Barnett et al. 2005, McMenamin et al. 2008).

The wetland temperature data revealed that there were not obvious site-specific differences, but there were some differences in daily temperature variation based on wetland size. Smaller wetlands tended to have greater daily fluctuation in temperature and tadpoles in smaller ponds are thus exposed to greater high and low extremes. Studies with Alaskan wood frogs have shown that tadpole development can be greatly influenced by small changes in temperature. For example, Herreid and Kinney (1967) found that, when temperatures were increased from 5.6°C to 10.0°C, tadpoles progressed from Witschi (1956) stage 0 to 20 (hatching) in almost a quarter of the time. Similar results were also observed in field studies in which tadpoles from the same ponds reached Witschi stage 30 ten days sooner in 1964 when pond temperatures averaged 3 – 6°C warmer compared to 1965 (Herreid and Kinney 1967). The authors also conclude that the egg to hatching stage was heavily influenced by temperature, but, once free-swimming, tadpoles can seek optimal temperatures and thus the effect of temperature on further development is somewhat diluted by other factors. Further, while development rate is

affected by water temperature, growth rate alone is influenced by other environmental factors including density such that the effect of temperature is not as clear (Smith-Gill and Berven 1979). In light of predicted effects of climate change on PPR wetlands, e.g., warmer temperatures and more severe drought, tadpoles in smaller wetlands may be forced to develop faster as a function of temperature increase and in response to wetland desiccation, which may in turn have indirect negative effects on their ability to gain mass to sustain themselves through metamorphic climax. Given the regional variation of wood frog growth and development rates and the subsequent differential responses to temperature changes, however, making broad, range-wide conclusions about the potential effect of wetland temperatures on wood frog tadpoles is difficult (Berven and Gill 1983).

3.4.2 Factors Influencing Tadpole Growth and Health

3.4.2.1 Tadpole Body Condition and Body Mass

Initial modeling attempts used body condition as the response variable to test potential effects of environmental variables on tadpole mass while accounting for snout-vent length (SVL). However, the only variable that had a stronger influence than a random number was Gosner stage. Body condition remained stable or slightly declined until metamorphic climax (GS 41-42) where body condition dropped precipitously. To test if there was any influence of additional variables on tadpole and metamorph growth alone, I ran BRT models using body mass as the response variable and found that while Gosner stage still had the greatest influence on mass, total dissolved solids, proportion of pesticides detected, ammonia concentration, and wetland surface area also had relative influence values greater than a random number. The effect of Gosner stage on mass matched that of the raw data (Fig. 3.1B) in that mass steadily increased until metamorphic climax where mass began to decline. This phenomenon is due to the consumption of energy reserves to complete metamorphic climax, including the emergence of forelimbs, resorption of the tail, and reconstruction of many organs from larval to adult forms (Orlofske and Hopkins 2009).

For total dissolved solids there was an apparent threshold of around 600-700 mg/L, above which there was a negative influence on body mass. Total dissolved solids are a known concern for tadpoles in the specific context of road salt chlorides and may cause mortality, weight loss, delayed metamorphosis, and malformations especially in chronic exposures (Sanzo and Hecnar

2006). However, high-salinity wetlands in this region of Saskatchewan tend to have sulfate salts (Rawson and Moore 1944, Hammer 1978, Last and Ginn 2005) and very few studies have been conducted using these as contaminants of concern. Elphick et al. (2011) studied the toxicity of sulfate on several freshwater organisms to propose water quality guideline levels and found that toxicity generally declined with increased water hardness, although this was not the case for the sole amphibian species tested. For moderately hard water (80 – 100 mg/L) and hard water (160 – 250 mg/L) they proposed guidelines of 644 mg/L and 725 mg/L sulfate, respectively. These values are similar to the apparent TDS threshold for mass in this study, but they aren't easily comparable given that the actual ion constituency and water hardness of the sampled wetlands are unknown.

The BRT model indicated that at around 0.01 proportion of pesticides detected within a wetland, there was a strong negative influence on body mass. Since the proportion of pesticides detected in each wetland was calculated as the number of pesticides detected (i.e., above limits of detection) out of the total number for which water samples were screened, the 0.01 proportion translates to 1.66 and 1.72 pesticides detected in 2017 and 2018, respectively. Of the twelve wetlands included in these models, five were not tested for pesticides including Burr6 and Colonsay6 in 2017, and Allan3, Burr11, and St. Denis6 in 2018. Of those that were tested, only Burr5 in 2017 and St. Denis2 in 2018 had no pesticides detected. Although there were no detections in the water sample collected, it is unlikely that Burr5 had absolutely no pesticide contamination in 2017 as there were detections in 2018, and I observed almost direct application of at least some early to mid-season pesticides to wetland vegetation while in the field in 2017. The sum concentration of pesticides did not appear to have an influence on mass, but detection of only one or two pesticides corresponds to low total concentrations (range 0.037 – 0.87 µg/L), likely well below known lethal effects thresholds, but within the range of some sublethal effects (Relyea 2004, Mann et al. 2009).

The most commonly detected pesticides in tadpole-containing wetlands were the herbicides methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-dichlorophenoxyacetic acid (2,4-D; see Section 2.3.1). Johnasson et al. (2006) found no effect of MCPA on *Rana temporaria* tadpole survival or growth during acute exposures, thus further supporting its low toxicity to amphibians as suggested by a $LC50_{120h}$ value of 3.6 g/L (Bernardini et al. 1996). Similarly, reported $LC50_{96h}$ values of 2,4-D are higher than the concentrations found in these

wetlands, although they do appear to range substantially from at least 8.05 mg/L to > 270 mg/L (Vardia et al. 1984, Morgan et al. 1996). Relyea (2005) also found no effect of 2,4-D on tadpoles, but all of these examples were, typically, single-contaminant lab toxicity tests. In general, pesticides can cause reduced larval growth (Howe et al. 2004, Mann et al. 2009, Lanctôt et al. 2014) and smaller size at metamorphosis can have negative consequences for individual survival and reproductive success as an adult (Berven and Gill 1983, Smith 1987). Given the low total concentrations of pesticides in these wetlands, it is unlikely that they alone are the sole factor in causing reduced tadpole mass. Mixtures of pesticides with other agricultural contaminants, like fertilizers, can have varying effects on tadpole growth (Relyea 2004, Mann et al. 2009, Smith et al. 2011). Further, there may be other factors contributing to the observed reduction in tadpole mass at wetlands with higher pesticide detections, including co-occurrence of unmeasured pesticides (e.g., glyphosate, Relyea 2005, Mann et al. 2009) or eutrophication. Eutrophication may increase tadpole exposure to parasites and simultaneous exposure to agricultural contaminants may increase likelihood of infection (Kiesecker 2002, Johnson and Sutherland 2003). Tadpoles dealing with pathogenic infection can also suffer from reduced mass (Kiesecker 2002).

Ammonia concentrations and wetland surface area also had an impact on tadpole and metamorph mass, but their effects appear rather marginal compared to the aforementioned variables and somewhat difficult to interpret. Both variables had fairly neutral effects except when ammonia concentrations were around 0.5 – 0.75 NH_3-N mg/L (0.61 – 0.91 NH_3 mg/L) and when wetland surface area was around 4000 – 6000 m^2, at which there were slightly positive influences on mass. Ammonia is known to be toxic to amphibians in acute tests, with reported $LC50_{96h}$ values of 0.42 to 1.9 NH_3 mg/L (see Mann et al. 2009). These overlap the values observed here that had slightly positive influences on mass, thus contradicting what may be expected, although there do appear to be species-specific tolerance levels (e.g., no effects on *Bufo americanus* embryos at 0.9 NH_3 mg/L, Jofre and Karasov 1999). One reason for this contradiction may be that the aforementioned effects of ammonia on body mass, based on the BRT model, are when the effects of all other variables on mass are held at their average such that, when ammonia concentrations interact with the effects of many other environmental variables, there may be positive or neutral effects. Ammonia can also act as a fertilizer which may increase algal abundance and consequently increase larval mass (Belden 2006). Finally,

given the transient nature of ammonia in freshwater systems, the concentrations reported in this study may not accurately represent the chronic, fluctuating concentrations to which these tadpoles are being exposed (Mann et al. 2009).

With respect to wetland surface area, the effect is similarly marginal. Positive influences of surface area on body mass may reflect the influence of hydroperiod on tadpole development overall. Wetlands that are prone to desiccate too quickly may force tadpoles to accelerate metamorphosis and limit the time available to grow in size before metamorphic climax (see Section 3.4.1 for further discussion on accelerated metamorphosis). Slightly larger wetlands may allow tadpoles more time to develop energy reserves before metamorphosis despite the greater potential for predators (e.g., fish) to establish in larger wetlands. Previous research has reported wood frog tadpoles in a variety of wetland sizes from 500 to almost 10,000 m^2 (Egan and Paton 2004).

The dissimilarities between the body condition and body mass BRT models reflect the differences in each metric's implication. The body condition metric is a way of assessing larval energy stores and health by measuring mass with respect to size (i.e., SVL), whereas body mass alone is simply assessing overall size since mass and SVL are linearly related (Fig. 3.1A). The models for both metrics accounted for the effects of development, or Gosner stage, but only mass was influenced by other variables. Lanctôt et al. (2014) reported similar discrepancies in which exposure to various glyphosate treatments had significant effects on tadpole mass but not on body condition, and vice versa, depending on Gosner stage. There is some concern of using tadpole body condition to assess energy stores due to its ability to be influenced by a number of larval and environmental factors including gut fill, body damage or deformity, sex, genetic variation, hydroperiod, temperature, and density (MacCracken and Stebbings 2012). As such, to assess larval health in terms of energy stores, using something like the scaled mass index (SMI) may be more insightful (MacCracken and Stebbings 2012). Nevertheless, both of these metrics, body condition and body mass, are frequently applied in anuran research and provide interesting comparisons here.

Both models in this study are also limited by their datasets. Due to the haphazard nature of how I selected wetlands to use for water quality and wetland habitat measurements in 2018, I did not include tadpoles or metamorphs collected from Colonsay6 or Colonsay16 in these models because they only had associated explanatory data for land use, surface area, and

ranavirus presence or absence, and lacked all the other water quality and wetland habitat variables used for other modelling. I felt that this was too limited a dataset for these individuals to warrant including them in the models. So, while there are many tadpole and metamorph individuals included in each model (292), they are sourced only from twelve wetlands in total and the explanatory data associated with individuals are, therefore, highly repetitive.

3.4.2.2 Tadpole Health: Neutrophil-to-Lymphocyte Ratios

Like the BRT model for body condition, only Gosner stage was more influential than random on tadpole and metamorph N:L ratios, indicating no effects from the measured environmental variables and that this immune status indicator is rather dictated by metamorphosis. N:L ratios were generally stable until metamorphic climax, after which values dropped rapidly, indicating a decline in neutrophils and/or an increase in lymphocytes. This is similar to the observations made by Davis (2009) in bullfrog tadpoles. Both neutrophils and lymphocytes were abundant during early growth of tadpoles but at metamorphic climax counts of both declined with neutrophil counts declining more precipitously which would result in lower N:L ratios (Davis 2009). Overall, however, the range of N:L ratio values is similar to that reported from northern leopard frogs (*Lithobates pipiens*) collected from wetlands exposed to pesticides (Shutler and Marcogliese 2011). I found an abundance of unique neutrophils in many blood smears but these observations were mostly made in individuals collected at Burr; thus, the high maximum value of N:L ratios (8.0, see Table A-17). Little research has been done on amphibian white blood cells, especially with wood frog tadpoles, and I did not find similar neutrophil examples in references used to guide identification (Heatley and Johnson 2009, Forzán et al. 2016). Jordan and Speidel (1923, p. 381) did make mention of "special granulocytes (pseudo-eosinophils or neutrophils)", but it is unclear what made these particular leukocytes remarkable. After discussions with veterinary pathologists and others, I concluded that they were likely neutrophils with 1° granules (M. Forzán and M. Meachem 2018, pers. comm.). Primary granules are generally thought of as storage sites of toxic mediators that may be released to kill bacteria or other pathogens (Lacy 2006). Thus, my findings may indicate some level of pathogenic stress in these tadpoles and metamorphs and which may not have been accounted for by the measured explanatory variables. Besides the anomalous neutrophils, another caveat of this dataset, which may or may not have affected the outcome of the BRT, is that collecting blood from tadpoles and metamorphs was difficult. For tadpoles, I could typically

get enough blood from the tail, but there was frequently other fluid associated with these collections that may have had leukocytes circulating in other parts of the tadpole body rather than strictly peripheral blood flow. Second, it was very difficult to get blood from the tails of metamorphs and I often had to collect blood from the heart, and lymphocyte profiles may differ between cardiac and peripheral blood (Shutler et al. 2009, Shutler and Marcogliese 2011). Finally, compared to the BRT models for body condition and mass, the percent deviance explained through cross validation was much lower in the N:L model. This suggests that model performance may be improved either by increased sample size (more wetlands), the inclusion of other explanatory variables, or by better blood collection methods. Despite the null result from this model and the difficulties associated with sample collection, observations of a unique neutrophil warrant further investigation into the leukocyte profiles of wood frog tadpoles. These investigations may be more thoroughly assessed in lab settings to better control for confounding factors including environmental variables and handling stress, and the potential to use flow cytometry which may improve our classification of the unique neutrophils.

3.4.3 Conclusions and Further Implications

Despite the somewhat limited dataset and lack of environmental effects on either body condition or N:L ratios, I did find effects of several water quality variables on the mass of wood frog tadpoles and metamorphs. Depending on the variable, the effects are either stark or subtle and do not always reflect results that may have been expected (e.g., ammonia concentrations). It is unlikely that TDS and proportion of pesticides detected are the only explanation for reduced mass, but they stand out as important factors and provide impetus for further field-based research on the effects of agricultural land use on tadpole health. In particular, very little work has looked at the effects of naturally-occurring ions that make up TDS on tadpoles, other than effects of seawater (e.g., see Hopkins and Brodie 2015). There is evidence that amphibians may be able to adapt to salty conditions, but identifying thresholds to the wood frog's distribution in the naturally more saline wetlands of the PPR will improve our understanding of their ecology and their susceptibility to additional natural and anthropogenic stressors (Hopkins and Brodie 2015). Although effects observed in this study were only found for mass, not body condition or N:L ratios, they do suggest that tadpoles in some agricultural settings may be at a disadvantage. Frogs metamorphosing at smaller body size may be less able to evade predators (Beck and Congdon 2000), and may reach reproductive age later than their larger counterparts (Berven and

Gill 1983, Smith 1987). Overall, these results highlight the complexity of field studies wherein the effects of water quality, wetland habitat, and land use are all acting on individuals simultaneously and may have effects that are different from those observed in lab-based investigations (Lanctôt et al. 2014).

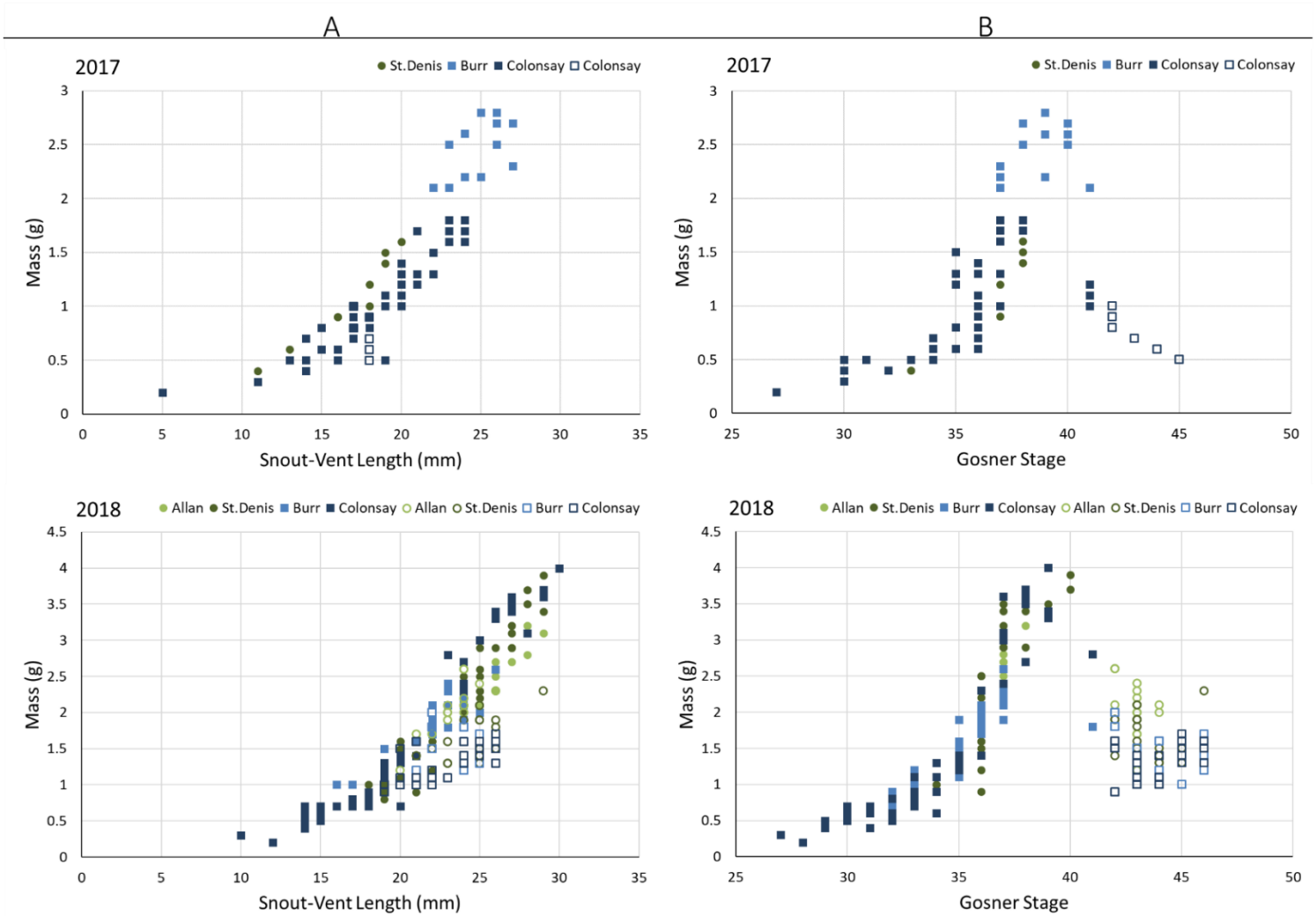

Figure 3.1. Relationships between mass and snout-vent length (panel A), and mass and Gosner stage (panel B), of wood frog tadpoles and metamorphs collected at four sites in 2017 and 2018. Circles represent grassland sites (Allan, St. Denis) and squares represent cropland sites (Burr, Colonsay). Solid points represent tadpoles and hollow points represent metamorphs.

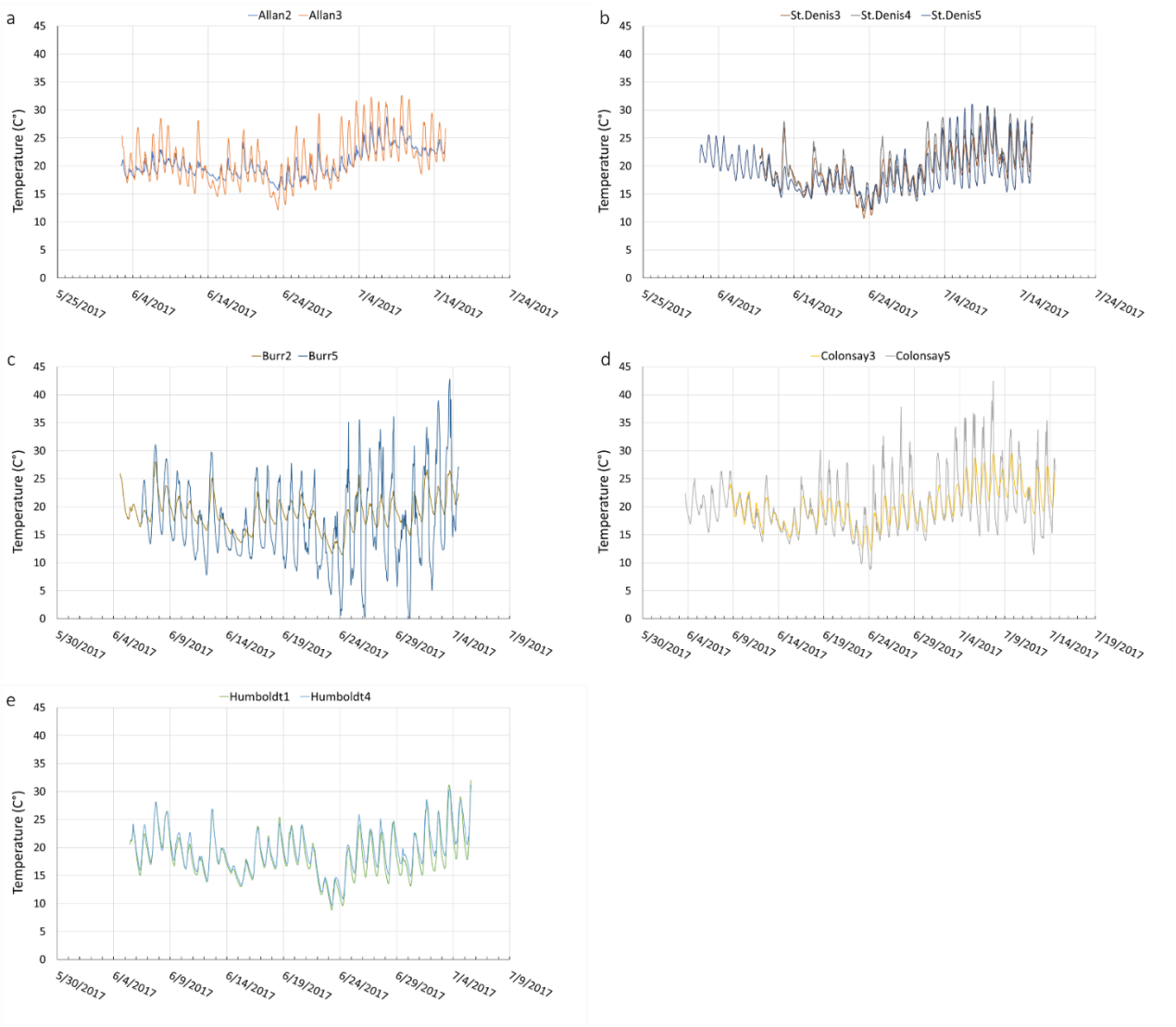

Figure 3.2. Temperature values collected from deployed HOBO loggers at two wetlands at every site in 2017, except at St. Denis where HOBOs were deployed at three wetlands. Grassland sites are Allan (a) and St. Denis (b) and cropland sites are Burr (c), Colonsay (d), and Humboldt (e).

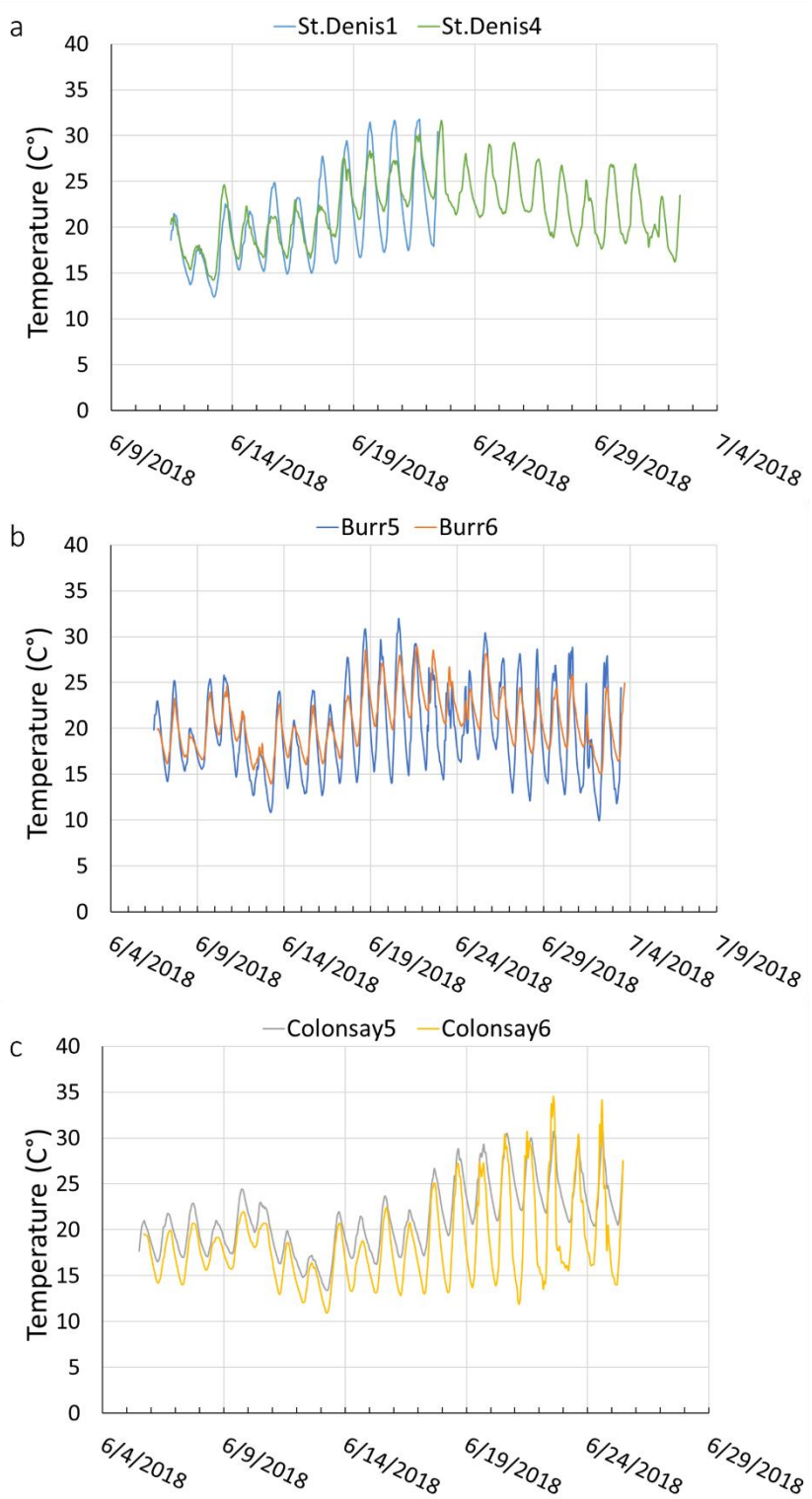

Figure 3.3. Temperature values collected from deployed HOBO loggers at two wetlands at three of five sites in 2018. St. Denis (a) is a grassland site and Burr (b) and Colonsay (c) are cropland sites.

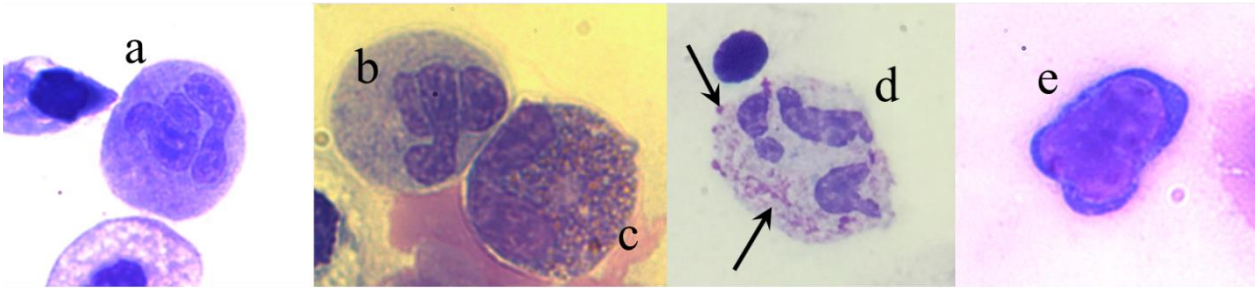

Figure 3.4. Examples of neutrophil and eosinophil white blood cells in the blood smears of wood frog tadpoles and metamorphs. Typical neutrophils (a, b) have segmented nuclei with lavender cytoplasm clear of any granules, eosinophils (c) have relatively large pink-magenta granules, and unique neutrophils (d) have small, pin-prick dark pink granules typically distributed patchily throughout the cytoplasm. Lymphocytes (e) have purple nucleus and very little cytoplasm. When these unique neutrophils were found in a blood smear, they were often very abundant. All photographs were taken at 1000x magnification by oil immersion.

Table 3.1. Parameters and evaluation metrices of the best performing BRT models for body condition (k), blood N:L ratios, and the top full (all variables) and simplified (only variables better than random; M.tc3.lr005.bf5.simp) BRT models for mass. *lr* refers to learning rate, *bf* to bag fraction, *tc* to tree complexity, *nt* to optimal number of trees, and dev to deviance.

Model Name	Response Variable	*lr*	*bf*	*tc*	*nt*	Mean Total Dev	Mean Residual Dev	Residual % Dev Explained	Estimated CV Dev	CV % Dev Explained
K.tc3.lr005.bf5	body condition (k)	0.005	0.5	3	1350	0.023	0.013	43.48	0.018	21.74
NL.tc3.lr001.bf5	N:L ratio	0.001	0.5	3	1650	0.121	0.093	23.14	0.107	11.57
M.tc3.lr005.bf5	mass	0.005	0.5	3	2750	0.598	0.037	93.81	0.067	88.80
M.tc3.lr005.bf5.simp	mass	0.005	0.5	3	4200	0.598	0.037	93.81	0.055	90.80

Table 3.2. Relative influence values of predictor variables in the BRT models containing all variables for body condition (k, K.tc3.lr005.bf5), blood N:L ratio (NL.tc3.lr001.bf5), and mass (M.tc3.lr005.bf5), and for the mass model containing only those variables that performed better than random (M.tc3.lr005.bf5.simp). See Table A-1 for variable descriptions.

K.tc3.lr005.bf5		NL.tc3.lr001.bf5		M.tc3.lr005.bf5		M.tc3.lr005.bf5.simp	
Variable	Relative Influence	Variable	Relative Influence	Variable	Relative Influence	Variable	Relative Influence
GS	51.04	GS	21.24	GS	29.79	GS	35.04
RandNum	20.42	RandNum	17.97	TDS	21.29	TDS	32.19
DP	5.47	VegBuffWidth	11.58	PestDet	19.90	PestDet	21.90
PestDet	4.33	PropNatural	8.84	DNAmm	7.00	DNAmm	8.33
SurfaceArea	3.66	DNAmm	7.11	SurfaceArea	4.03	SurfaceArea	2.54
DNAmm	3.22	PropWater	5.87	RandNum	3.71		
PestSumConc	1.43	FillCode	4.56	PestSumConc	3.15		
VegBuffWidth	1.21	pH	2.51	DO	1.77		
Year	1.07	Situation	1.86	PropWater	1.57		
FillCode	0.92	PropPasture	1.83	Situation	1.01		
CPUE	0.88	AlgaeCover	1.67	CPUE	0.87		
Chla	0.70	SurfaceArea	1.44	Turbidity	0.62		
PropPasture	0.58	PropCrops	1.43	VegBuffWidth	0.52		
pH	0.58	PestDet	1.33	pH	0.50		
DO	0.52	DP	1.29	Chla	0.49		
Classification	0.51	Year	1.22	Classification	0.42		
Surrounding Land	0.49	TN	1.11	PropUrban	0.42		
Turbidity	0.49	TDS	1.09	FillCode	0.37		
DN	0.47	DO	0.76	PropExposed	0.30		
TDS	0.45	Surrounding Land	0.76	DP	0.30		
TN	0.42	DN	0.73	TN	0.29		
AlgaeCover	0.28	Turbidity	0.67	Year	0.29		
Situation	0.28	Chla	0.67	AlgaeCover	0.26		
PropUrban	0.21	CPUE	0.60	RoadDist	0.24		
PropExposed	0.15	RVeDNA	0.51	PropNatural	0.24		
RoadDist	0.14	RoadDist	0.34	DN	0.21		
PropNatural	0.05	PropUrban	0.31	Surrounding Land	0.19		
VegCover	0.01	Classification	0.24	PropPasture	0.15		
RVeDNA	0.01	VegCover	0.22	PropCrops	0.06		
Connectivity	0.00	PestSumConc	0.14	RVeDNA	0.05		
Fish	0.00	PropExposed	0.06	VegCover	0.01		
PropWater	0.00	Connectivity	0.05	Connectivity	0.00		
PropCrops	0.00	Fish	0.00	Fish	0.00		

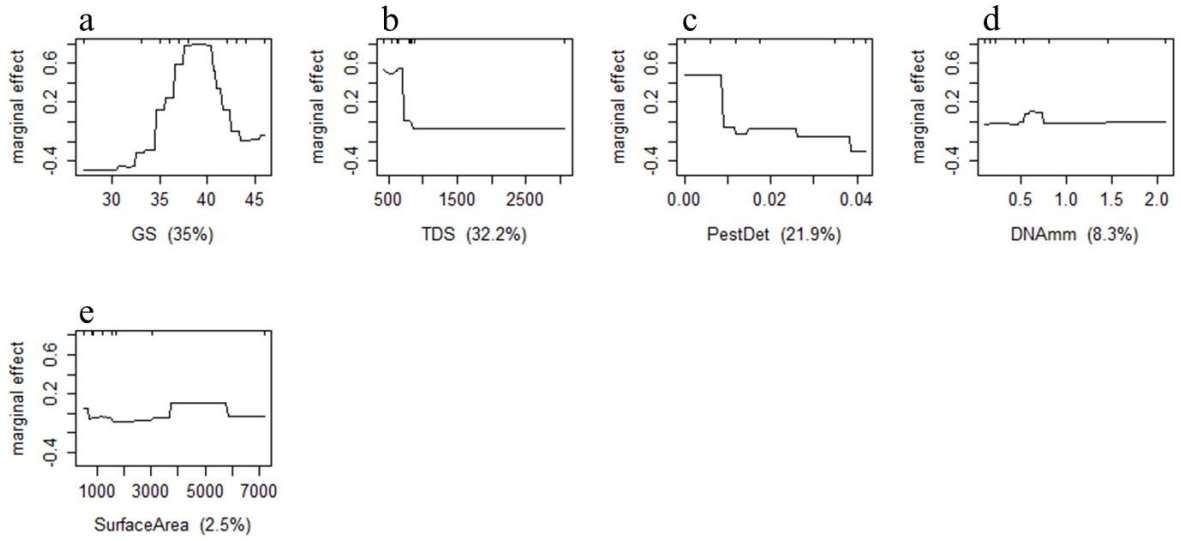

Figure 3.5. Partial dependence plots (a-e) for the simplified mass BRT model showing the five variables with relative influence greater than a random number: GS – Gosner stage, TDS – total dissolved solids (mg/L), PestDet – proportion of pesticides detected, DNAmm – ammonia (mg/L), and SurfaceArea – wetland surface area (see Table A-1 for further detail). Marginal effect is the effect of the variable of interest on tadpole or metamorph mass (g) while the effects of the remaining variables are held at average. Percentages in parentheses are the relative influence of the respective variable. Rug plots on the top edge of each individual plot illustrate the distribution of sampling values in deciles (Elith et al. 2008).

CHAPTER 4: RESEARCH SYNTHESIS

4.1 SUMMARY OF FINDINGS

The overall goal of this project was to evaluate the impact of agricultural land use in the Prairie Pothole Region (PPR) on wood frog presence and health. This was divided into two main objectives.

4.1.1 Objective 1

The first objective of this research was to assess wood frog presence using environmental DNA (eDNA) at wetlands across an agricultural gradient and to relate it to environmental variables. Tangentially, I sought to identify effects of agricultural activity on wood frog habitat including measures of water quality, wetland habitat, and land use or cover. There were several key findings from this work. With respect to wood frog detection, eDNA was more successful than traditional observation methods. There was also seasonal variation in eDNA detection for both wood frogs and ranavirus in that detections were greater in the summer (June) than in the spring (May). Finally, and in support of my hypothesis, I found that wood frog detection did not differ between grassland and cropland sites.

The BRT models found that there was a combined influence of habitat variables across multiple scales on wood frog presence in the PPR. Primarily, variables that affected whether or not wood frogs were likely to be detected in a wetland included those related to wetland productivity (dissolved phosphorus, dissolved nitrogen, chlorophyll a), vegetation buffer width, and the proportion of the surrounding landscape that was also composed of water or wetland (Fig. 4.1). While the importance of each of these variables has been reported previously in the literature, their combined significance has been less emphasized. Additional variables that were more important than random include total dissolved solids (TDS), and catch-per-unit-effort (CPUE) of tadpoles – an index of tadpole density.

Of the 32 environmental variables examined several of them differed significantly among sites, but few of them showed differences which could be clearly distinguished between grassland and cropland locations. Those with the strongest differentiation between site types were the land use variables. In the field I observed evidence of nearby agricultural and cattle or bison activity at both grassland sites so they were not ‘pristine’ although they were certainly less impacted than the cropland sites. These findings highlight the modified nature of otherwise

pristine areas and support the eDNA results which showed no difference in wood frog presence between sites.

The results of the research performed to address this objective have supported and expanded upon pre-existing work done on amphibian occupancy in altered habitats. In particular, this work provides new data on wood frog ecology in the PPR where less wood frog research has been performed compared to more forested regions of the species' vast range (Guerry and Hunter 2002, Houlahan and Findlay 2003, Porej et al. 2004).

4.1.2 Objective 2

The second objective of this research was to assess tadpole and metamorph health using morphometrics (body condition and body mass) and neutrophil to lymphocyte (N:L) ratios, a measure of immune system stress. The key findings from this work include the lack of effects of environmental variables on either body condition or N:L ratios, but the effects of several variables, primarily related to water quality, on tadpole and metamorph mass (Fig. 4.2). These results provided mixed support for my predictions that size and health would be reduced at agricultural sites. For body condition and N:L ratios, the only variable that was more influential than random was Gosner stage, i.e., development. However, I did observe leukocytes in some individuals which, to my knowledge, have not been described before in anuran blood work. I termed these 'unique neutrophils' characterized by dark pink granules that were generally very small but appeared to occasionally aggregate into clumps, and varied from sparse to abundant among different cells.

The BRT model for body mass presented a different story in that, besides Gosner stage, there were several other variables more influential than random including TDS, proportion of pesticides detected, ammonia concentration, and wetland surface area. Of these, TDS and pesticides exhibited a negative effect on tadpole and metamorph mass compared to the other variables which had more subtle effects. This presents an interesting contrast with body condition as it suggests that mass for a given body length (snout-vent length, SVL) was not affected by TDS or pesticides, but that tadpole and metamorph size declined as a whole. That is, when mass declined so did SVL such that the individual tadpole or metamorph was smaller overall rather than 'small for its size' (Fig. 4.2).

Although there are several null results from the work done to address this second objective, they are still important. The results from the body condition and N:L ratio results

indicate that the tadpoles and metamorphs may not necessarily be less healthy in wetlands at agriculturally intensive sites, but they may be smaller. Size at metamorphosis can have important implications for frogs as juveniles and adults in terms of survival and fecundity (Berven and Gill 1983, Smith 1987, Beck and Congdon 2000), which could have population level effects if conditions are severe enough. These findings also add to the bigger question of 'how effective are lab-based toxicity tests at predicting real-world impacts?' and they appear to support the notion that lab-based studies may overestimate the impact of contaminants on frog populations. The identification of a unique leukocyte merits further study of tadpole and metamorph leukocyte profiles. Finally, and as with the previous objective, this work also contributes to our understanding of wood frog ecology in altered landscapes of the PPR.

4.2 GOING FORWARD

The results of this research project contribute to herpetology in general and to wood frog ecology specifically in a part of their range where it is less well described. While there are wood frogs present in agriculturally impacted wetlands, there are several stressors that may negatively influence both their presence on the landscape and their size at the tadpole and metamorph stages. These include aspects of water quality, wetland-specific habitat, and habitat heterogeneity at the landscape level. As discussed in Chapter 1, agricultural activities are one of the greatest threats to this regional herpetological hot spot in Canada (Lesbarrères et al. 2014). More broadly speaking, wetland habitat is also threatened by agriculture and as the province of Saskatchewan seeks to improve enforcement of wetland protection policy (Pattison-Williams et al. 2018), this work is timely in its ability to inform such legislation.

Given the large presence of wetland habitat and its importance environmentally and socio-economically, Canada was one of the first nations to enact federal legislation to protect wetlands, namely the Federal Policy on Wetland Conservation (FPWC; GC 1991). The FPWC has jurisdiction only on federal lands, but also seeks to guide and "promote the conservation of Canada's wetlands to sustain their ecological and socio-economic functions, now and in the future" (GC 1991, p. 5) by encouraging and working with provincial governments, indigenous peoples, international agreements (e.g., the North America Waterfowl Management Plan), and non-governmental organizations (e.g., Ducks Unlimited). Despite some efforts to curb wetland loss in North America, for example through the North American Waterfowl Management Plan of 1986, loss and degradation continue, albeit at a slower rate than in previous centuries (Bartzen et

al. 2010, Golden et al. 2017). Geographically isolated wetlands in particular, like those of the PPR, have been more easily ignored because their connection to downstream ecosystems is difficult to visualize (Golden et al. 2017). The federal legislation has strong objectives and goals but there is a lack of standardization among provincial policies and no method for keeping inventory of wetlands or to monitor successful or failed attempts at mitigation or compensation (Rubec and Hanson 2009). This makes it impossible to know whether policy is enforced or if it is successful at conserving wetlands (i.e., no net loss; Rubec and Hanson 2009). Despite efforts to emulate Alberta's more prescriptive policy (GA 2013), Saskatchewan has yet to update its own and thus remains fairly weak without mitigation guidelines and with jurisdiction only over crown lands (Rubec and Hanson 2009). There are more advanced assessment methods and reports on wetland status in the United States using remote sensing techniques (Dahl 2011). There are also several incentive programs in place to encourage wetland preservation, replacement, and/or reestablishment, especially in agricultural settings (e.g., the Conservation Reserve Program). These programs are useful in that they can result in small, localized gains of freshwater wetland area in agricultural systems, but they are often voluntary and thus are in competition with fluctuating crop prices such that when crop prices are particularly high, farmers are less likely to preserve fields for wetland conservation (Dahl 2011). Thus, declines in wetland area in agricultural regions, like the Dakotas, remain of heightened concern (Dahl 2011).

As this research has shown, wetland policy should address multiple levels of wetland habitat, from the pond to the landscape level. Preventing excessive nutrient and pesticide contamination would likely have positive influences on wood frog presence and health. Preserving vegetation buffers around wetlands will help preserve water quality (Haoukos et al. 2016, Main et al. 2017) and provide habitat for frogs (Semlitsch and Bodie 2003, Swanson et al. 2018). Finally, preventing wetland drainage and consolidation will provide a heterogeneous landscape with abundant sites for wood frogs to breed and act as stepping stones to nearby wetland and upland environments for foraging, overwintering, and migration (Egan and Paton 2004). Preserving each of these aspects of wetland habitat in the PPR will simultaneously provide some semblance of protection for various other wetland species including insects and birds.

This work contributes to our understanding of wood frog ecology, but also raises new questions. While reviewing the literature to inform my interpretation of the results, I found that

much of the wood frog research has been done in forested parts of its range. This is critical because there are large swaths of forested habitat in North America that have been lost to agriculture and/or urbanization. However, in the PPR where the predominant land cover is prairies and grasslands, conversion to agriculture is indeed different and destructive but, compared to loss of forested habitat, the alteration appears less drastic. Hence the finding that water and wetland cover on the landscape was important in predicting wood frog presence rather than forest or grassland cover, although natural cover was positively correlated with water. This suggests there may be a difference in the ecology of wood frogs throughout their range and makes one wonder if wood frogs in grassland habitat may be better able to adapt or persist amid agricultural conversion compared to populations which have evolved in forested landscapes. Expanding this research to include more sites throughout the prairies and then conducting identical work in other landscapes of the wood frog's range (e.g., forested, montane, tundra) using BRT models may help elucidate regional differences in the species' ecology. Improved understanding of regional differences, if they exist, will then better inform management and conservation efforts for the wood frog.

Given the relatively short duration of this project (two field seasons), I was unable to look at broad effects of climate on wood frog presence. In both years the study region experienced relatively dry conditions and I found several depressions that were dry even in the spring; in wet years these are likely ephemeral ponds that provide suitable breeding habitat (SRC 2018, WSA 2018). Although I did observe persistent flooding at some wetlands left over from a recent (2012-2014) wet period in the PPR, this was at large terminal wetlands. Many ephemeral wetlands were either dry through the spring and summer months or desiccated before summer's end. Wetland occupancy by wood frogs for breeding is known to be influenced by precipitation, and breeding cycles may track wet and dry cycles in the PPR (Donald et al. 2011). These ephemeral wetlands are more likely to fluctuate hydrologically in response to short-term, intra-annual climate and are also at greater risk of being lost to agriculture than more permanent wetlands, thus threatening habitat availability for wood frogs (Zhang et al. 2009, Bartzen et al. 2010).

With regard to tadpole and metamorph health, this work contributes to the collective comparison between lab and field research, but also introduces new questions. Of particular note here is the presence of unique neutrophils. Although I found no evidence for immune system

stress, at least in response to the variables I measured, the unique neutrophil, in and of itself, may be indicative of stress either from the environment or through metamorphosis. Further work on wood frog hematology, specifically with tadpoles, may improve our understanding of the changes and stress that tadpoles experience during metamorphosis.

With this work, I met my objectives and found support for some, but not all, of my hypotheses. In all, these efforts contribute new knowledge to our understanding of wood frog ecology in a rather unique part of its range. They also help provide a foundation for further investigations into wood frog ecology, particularly in the Northern Great Plains, and for future research on how different amphibian species may respond to agricultural stress at multiple spatio-temporal levels and in different landscapes. These kinds of comparative results will be important for informing management, conservation, and policy-related decisions.

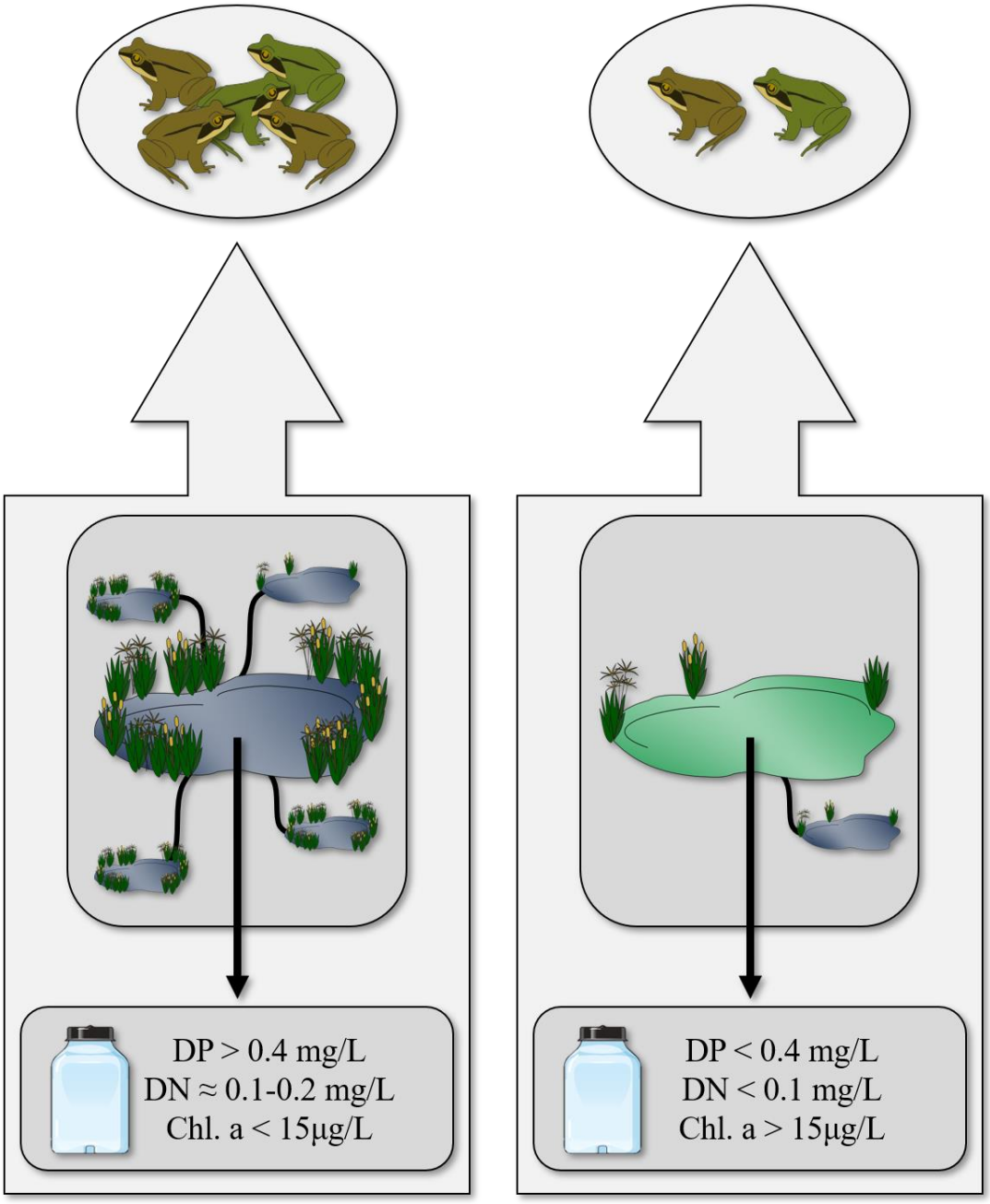

Figure 4.1. A graphical summary of some of the key results from Objective 1 (see Section 4.1.1) illustrating that, although detection of wood frogs did not differ between site types (grassland or cropland) a number of factors at different scales influenced detection likelihood (shown as more or less frogs). Positive wood frog detection was associated with greater proportion of water and wetlands on the landscape, larger vegetation buffers, higher dissolved phosphorus (DP), a narrow range of dissolved nitrogen (DN), and lower chlorophyll a (Chl. a) concentrations.

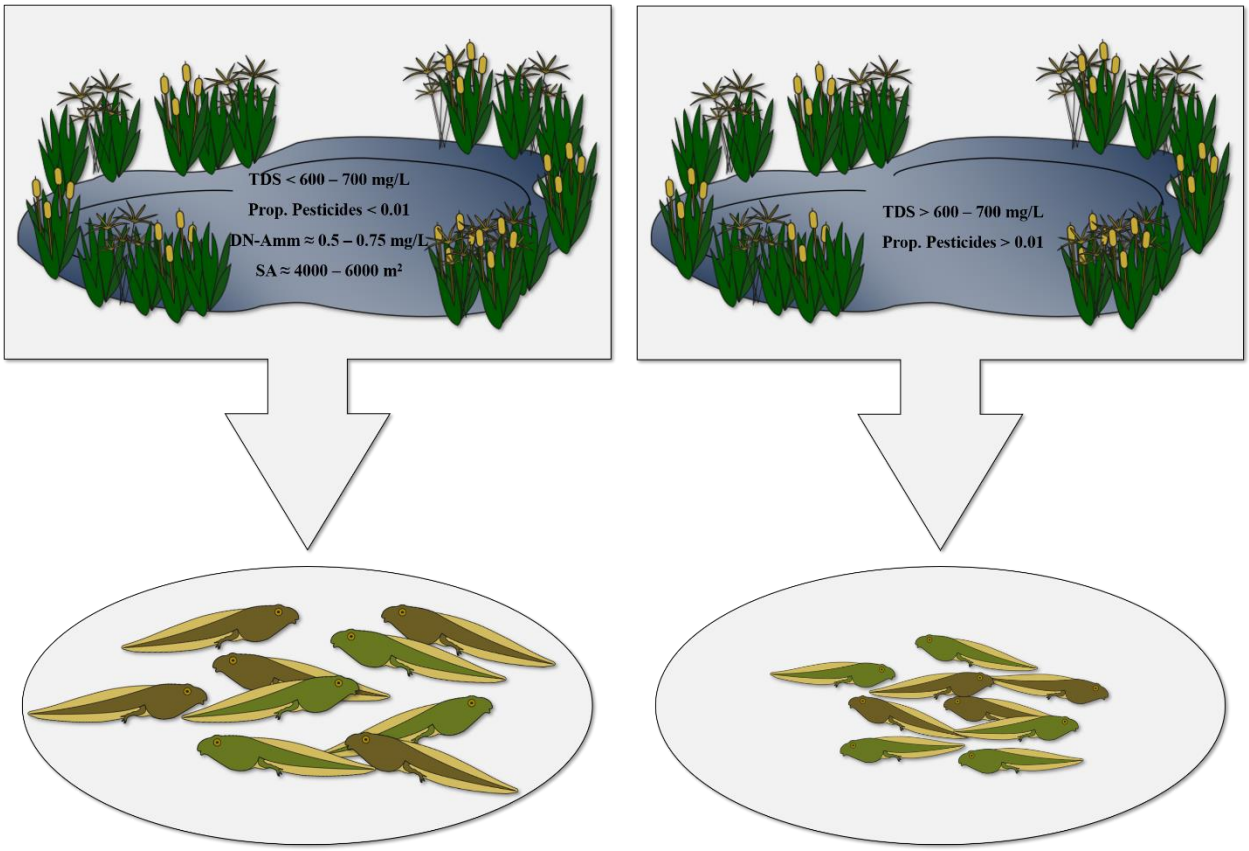

Figure 4.2. A graphical summary of some of the key results from Objective 2 (see Section 4.1.2) illustrating the effects of certain water quality variables on tadpole (and metamorph, not shown here) mass. Concentrations of total dissolved solids (TDS) greater than approximately 600 – 700 mg/L and proportion of pesticides detected greater than 0.01 were associated with lower mass. Ammonia concentrations (DN-Amm, NH_3-N mg/L) around 0.5 – 0.75 mg/L and wetland surface area between 4000 – 6000 m^2 had small positive effects on mass.

APPENDIX

Table A-1. Descriptions of land use, habitat, water quality, pesticide, and disease variables measured. Words in parentheses are the variable name used to run models.

Variable	Description
Land Use[a]	
Crops (PropCrops)	Includes any land classified as barley, oats, spring wheat, canola/rapeseed, lentils, soybeans, or peas.
Pasture and forage (PropPasture)	Land that is periodically cultivated for livestock as pasture including alfalfa, clover, etc.
Natural (PropNatural)	Any land that is classified as coniferous, broadleaf or mixedwood forest, shrubland (woody vegetation of low height), or grassland (native herbaceous vegetation).
Urban and developed (PropUrban)	Includes land that is developed and associated vegetation such as roads, railways, buildings, urban or industrial areas, etc.
Exposed and barren land (PropExposed)	Non-vegetated, non-developed land, excludes fallow agricultural land.
Water and wetlands (PropWater)	Includes land classified as a water body or wetland including lakes, streams, reservoirs, marshes, sloughs, bogs, etc.
Wetland Habitat Features	
Surface Area (SurfaceArea)	Surface area of wetland estimated with ArcMap (m^2)
Bison/cow (Bovid)	Presence or absence of them or sign (tracks in wetland margin).
Fish (Fish)	Presence or absence determined during VES or dipnet sweeps. Fish observed in either 2017 or 2018 were assumed to also be present in the other year.
Connectivity (Connectivity)	Whether or not the wetland was connected to an adjacent one
Basin fill code (FillCode)	Visual estimate of fullness of wetland: 0 = dry, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = >76%
Crop type/surrounding land use (SurroundingLand)	Visual determination of surrounding crop type or land use
Road distance (RoadDist)	Distance to nearest road (includes graded roads) determined using ArcMap (m)
Wetland situation (Situation)	Refers to hydrogeomorphology and the wetland's position in the landscape. 1 = Isolated: high areas, often temporary, rarely overflow; 2 = Overflow: receives water from surrounding area, in high areas and temporary but may overflow; 3 = Channel: may receive water from surrounding land and other wetlands, may overflow; 4 = Terminal: in low areas and serves as an endpoint of

	local drainage, can't overflow; 5 = Junction: two or more contiguous wetlands at same elevation, exist in wet or flooded years.
Wetland vegetation cover (VegCover)	Refers to vegetation cover in the wetland. 1 = stands of emergent vegetation throughout the wetland with < 5% open water or soil; 2 = scattered stands of emergent vegetation in the wetland but with 5-95% open water or soil; 3 = the central portion of the wetland is open water or soil with a band of vegetation on the edge; 4 = wetland is >95% open water or bare soil.
Vegetation buffer width (VegBuffWidth)	Average vegetative zone width of a wetland (m). In the field, measurements of wet meadow and shallow marsh zones were taken at the four cardinal directions of each wetland, combined and then averaged. Wet meadow was identified as including wetland vegetation of lower height, but also included willow shrubs; was frequently missing in wetlands at cropland sites. Shallow marsh was identified as wetland vegetation of intermediate height although often included cattails (*Typha* spp.) with shallow water or saturated soil through mid-summer in normal years.
Algae cover (AlgaeCover)	Visual estimated percent of the wetland surface the consisted of algae and/or duckweed compared to open water.
Wetland Classification (Classification)	Classification of wetland permanency, including visual assessment of wetland capacity and vegetative zones. 2 = temporary wetland with central zone composed primarily of wet meadow vegetation; 3 = seasonal, central zone comprised of shallow marsh vegetation; 4 = semi-permanent, central zone contains deep-marsh vegetation; 5 = permanent, central zone comprised of open water, may have submerged vegetation but is not emergent.
Water Quality	
Dissolved oxygen (DO)	DO, mg/L
pH (pH)	
Total dissolved solids (TDS)	TDS, mg/L
Turbidity (Turbidity)	FNU
Chlorophyll a (Chla)	µg/L
Nitrate (DN)	Dissolved nitrogen; mg/L N
Phosphate (DP)	Dissolved phosphorus; mg/L P
Ammonia (DNAmm)	mg/L N
Total nitrogen (TN)	mg/L N
Total Phosphorus (TP)	mg/L P, not used in models because highly correlated with DP
Pesticides	

Proportion detected (PestDet)	Proportion of pesticides detected of those scanned for, including four neonicotinoids, at each sampled wetland. (166 scanned for in 2017, 172 in 2018).
Sum concentration (PestSumConc)	Total concentration of any detected pesticides, including four neonicotinoids, at each sampled wetland.'
Disease	
Ranavirus (RVeDNA)	Presence or absence of ranavirus determined via eDNA methods.
CPUE (CPUE)	Catch per unit effort. Number of tadpoles per dipnet sweep.

[a] For greater detail on land use descriptions see the AAFC Annual Crop Inventory Data Product Specifications at https://open.canada.ca/data/en/dataset/ba2645d5-4458-414d-b196-6303ac06c1c9

Table A-2. Comparison of wood frog detection using traditional and eDNA methods, and eDNA detection of ranavirus, including number of positive samples and positive replicates in summer 2017.

	Wood Frog				Ranavirus		
Wetland	Traditional Detection	eDNA Detection	Positive Water Samples	Positive qPCR Replicates	eDNA Detection	Positive Water Samples	Positive qPCR Replicates
Allan 1	+	-	0	0	-	0	0
Allan 2	-	-	0	0	-	0	0
Allan 3	+	-	0	0	-	0	0
Allan 4	+	-	0	0	+	2	2
Burr 1	-	+	3	9	-	0	0
Burr 2	-	-	0	0	-	0	0
Burr 5	+	+	3	9	-	0	0
Burr 6	+	+	2	6	-	0	0
Burr R1S	-	-	0	0	-	0	0
Burr R2N	-	-	0	0	-	0	0
Colonsay 1	-	+	1	1	-	0	0
Colonsay 2	+	-	0	0	-	0	0
Colonsay 3	-	+	1	2	-	0	0
Colonsay 4	-	+	1	1	-	0	0
Colonsay 5	+	+	3	9	-	0	0
Colonsay 6	+	+	3	9	-	0	0
Humboldt 1	-	-	0	0	-	0	0
Humboldt 2	-	+	2	3	-	0	0
Humboldt 3	-	-	0	0	-	0	0
Humboldt 4	-	+	1	1	-	0	0
Humboldt 5	-	-	0	0	-	0	0
St.Denis 1	+	+	3	9	-	0	0
St.Denis 2	-	+	2	4	+	2	6
St.Denis 3	+	-	0	0	+	1	3
St.Denis 4	+	+	1	2	+	1	3
St.Denis 5	-	+	1	2	-	0	0

Table A-3. Comparison of wood frog detection using traditional and eDNA methods, and eDNA detection of ranavirus, including number of positive samples and positive replicates in spring 2018.

Wetland	Wood Frog				Ranavirus		
	Traditional Detection	eDNA Detection	Positive Water Samples	Positive qPCR Replicates	eDNA Detection	Positive Water Samples	Positive qPCR Replicates
Allan 1	-	-	0	0	-	0	0
Allan 2	-	-	0	0	-	0	0
Allan 3	+	+	3	5	-	0	0
Allan 4	-	-	0	0	-	0	0
Allan 8	-	+	2	3	-	0	0
Allan 10	-	-	0	0	-	0	0
Burr 1	-	-	0	0	-	0	0
Burr 2	-	-	0	0	-	0	0
Burr 5	+	+	2	5	-	0	0
Burr 6	+	-	0	0	-	0	0
Burr 9	+	-	0	0	-	0	0
Burr 10	-	+	2	4	-	0	0
Burr 11	-	+	3	8	-	0	0
Burr 12	-	+	3	7	-	0	0
Burr 17	-	-	0	0	-	0	0
Burr R1S	-	-	0	0	-	0	0
Burr R2N	-	-	0	0	+	1	1
Colonsay 1	-	-	0	0	-	0	0
Colonsay 2	-	+	1	3	-	0	0
Colonsay 3	+	-	0	0	-	0	0
Colonsay 4	-	+	2	6	-	0	0
Colonsay 5	+	+	3	6	-	0	0
Colonsay 6	-	+	2	3	-	0	0
Colonsay 7	-	+	1	1	-	0	0
Colonsay 8	-	-	0	0	-	0	0
Colonsay 9	-	-	0	0	-	0	0
Colonsay 10	-	-	0	0	-	0	0
Colonsay 11	-	-	0	0	-	0	0
Colonsay 12	-	+	2	5	-	0	0
Colonsay 13	+	+	3	8	-	0	0
Colonsay 14	-	-	0	0	-	0	0
Colonsay 16	+	+	3	7	-	0	0
Colonsay 17	-	+	2	3	-	0	0
Colonsay 19	-	+	2	4	-	0	0

Colonsay 20	+	+	1	3	+	1	2
Humboldt 1	-	-	0	0	-	0	0
Humboldt 2	-	-	0	0	-	0	0
Humboldt 3	-	-	0	0	-	0	0
Humboldt 4	-	-	0	0	-	0	0
Humboldt 5	-	-	0	0	-	0	0
Humboldt 6	-	+	1	2	-	0	0
Humboldt 7	-	+	2	4	-	0	0
Humboldt 8	-	-	0	0	-	0	0
Humboldt 13	-	-	0	0	-	0	0
Humboldt 18	-	+	2	3	-	0	0
Humboldt 19	-	-	0	0	-	0	0
Humboldt 20	-	-	0	0	-	0	0
Humboldt 21	-	-	0	0	-	0	0
Humboldt 22	-	+	1	2	-	0	0
Humboldt 24	-	-	0	0	+	1	3
Humboldt 25	-	-	0	0	-	0	0
Humboldt 27	-	-	0	0	-	0	0
Humboldt 28	-	+	1	1	-	0	0
Humboldt 33	-	+	1	3	-	0	0
Humboldt 36	-	-	0	0	-	0	0
Humboldt 41	-	-	0	0	-	0	0
Humboldt 100	-	-	0	0	-	0	0
St.Denis 1	-	-	0	0	-	0	0
St.Denis 2	-	-	0	0	-	0	0
St.Denis 3	-	-	0	0	-	0	0
St.Denis 4	-	-	0	0	-	0	0
St.Denis 5	+	+	1	2	-	0	0
St.Denis 6	+	-	0	0	-	0	0
St.Denis 7	-	-	0	0	-	0	0
St.Denis 8	-	-	0	0	-	0	0
St.Denis 9	-	-	0	0	-	0	0
St.Denis 12	-	+	1	3	-	0	0
St.Denis 18	-	-	0	0	-	0	0
St.Denis 19	-	-	0	0	-	0	0
St.Denis 20	-	-	0	0	-	0	0
St.Denis 23	-	-	0	0	-	0	0

Table A-4. Comparison of wood frog detection using traditional and eDNA methods, and eDNA detection of ranavirus, including number of positive samples and positive replicates in summer 2018. Traditional detections are an accumulation and include those made in spring 2018.

	Wood Frog				Ranavirus		
Wetland	Traditional Detection	eDNA Detection	Positive Water Samples	Positive qPCR Replicates	eDNA Detection	Positive Water Samples	Positive qPCR Replicates
Allan 1	-	-	0	0	+	3	5
Allan 2	-	+	1	2	+	1	2
Allan 3	+	+	3	9	+	1	2
Allan 4	-	+	1	2	-	0	0
Allan 8	+	-	0	0	-	0	0
Allan 10	-	+	1	2	-	0	0
Burr 1	-	+	1	3	-	0	0
Burr 2	-	-	0	0	-	0	0
Burr 5	+	+	3	9	-	0	0
Burr 6	+	+	2	5	-	0	0
Burr 9	+	+	2	4	-	0	0
Burr 10	-	+	2	5	-	0	0
Burr 11	+	+	3	9	-	0	0
Burr R1S	-	-	0	0	-	0	0
Burr R2N	-	+	1	3	-	0	0
Colonsay 1	-	-	0	0	-	0	0
Colonsay 2	+	+	1	1	-	0	0
Colonsay 3	+	+	2	4	-	0	0
Colonsay 4	+	+	3	6	+	3	8
Colonsay 5	+	+	3	9	-	0	0
Colonsay 6	+	+	3	9	+	1	2
Colonsay 7	-	-	0	0	-	0	0
Colonsay 8	-	-	0	0	+	1	3
Colonsay 9	-	+	1	3	+	3	8
Colonsay 10	-	-	0	0	-	0	0
Colonsay 12	-	+	2	5	+	2	6
Colonsay 13	+	+	1	2	-	0	0
Colonsay 14	-	+	3	9	+	1	2
Colonsay 16	+	+	3	9	+	1	3
Colonsay 17	-	-	0	0	-	0	0
Colonsay 19	-	+	3	9	-	0	0
Colonsay 20	+	+	1	3	+	2	4
Humboldt 1	-	+	1	2	+	3	4
Humboldt 2	-	+	1	3	+	2	4

Humboldt 3	-	-	0	0	+	2	6
Humboldt 4	-	-	0	0	-	0	0
Humboldt 5	-	+	2	5	-	0	0
Humboldt 6	-	+	3	9	+	3	9
Humboldt 7	-	+	2	5	+	3	9
Humboldt 13	-	-	0	0	-	0	0
Humboldt 18	-	-	0	0	-	0	0
Humboldt 19	-	-	0	0	-	0	0
Humboldt 20	-	-	0	0	+	1	2
Humboldt 21	-	+	1	1	-	0	0
Humboldt 22	-	+	3	6	-	0	0
Humboldt 24	-	+	1	2	+	3	8
Humboldt 27	-	+	1	2	-	0	0
Humboldt 28	-	+	3	8	+	3	9
Humboldt 36	-	-	0	0	-	0	0
Humboldt 41	+	+	3	8	-	0	0
Humboldt 100	-	-	0	0	-	0	0
St.Denis 1	-	-	0	0	-	0	0
St.Denis 2	+	+	1	3	-	0	0
St.Denis 3	-	+	1	2	+	3	8
St.Denis 4	-	+	1	3	+	3	7
St.Denis 5	+	+	3	9	+	2	2
St.Denis 6	+	+	3	6	-	0	0
St.Denis 7	-	+	1	1	+	3	9
St.Denis 8	-	+	1	3	-	0	0
St.Denis 9	-	+	1	2	+	3	9
St.Denis 12	-	+	2	5	-	0	0
St.Denis 18	-	+	1	2	-	0	0
St.Denis 19	-	+	1	3	+	3	8
St.Denis 20	-	-	0	0	+	3	8
St.Denis 23	-	-	0	0	-	0	0

Table A-5. All pesticides screened for in 2017 and 2018 and their associated function.

2017	2018	Function
Imidacloprid	Imidacloprid	Neonicotinoid
Thiamethoxam	Thiamethoxam	Neonicotinoid
Clothianidin	Clothianidin	Neonicotinoid
Acetamiprid	Acetamiprid	Neonicotinoid
2,4-Dichlorophenoxyacetic acid	2,4-Dichlorophenoxyacetic acid	Herbicide
4-(2,4-dichlorophenoxy)butyric acid	4-(2,4-dichlorophenoxy)butyric acid	Herbicide
2,4-Dichlorophenol	2,4-Dichlorophenol	Herbicide
Alachlor	Alachlor	Herbicide
Aldrin	Aldrin	Insecticide
Allidochlor	Allidochlor	Herbicide
Atrazine	Atrazine	Herbicide
Azinphos-methyl	Azinphos-methyl	Insecticide
Azoxystrobin	Azoxystrobin	Fungicide
Benalaxyl	Benalaxyl	Fungicide
Benfluralin	Benfluralin	Herbicide
Bentazon	Bentazon	Herbicide
Benzoylprop-Ethyl	Benzoylprop-Ethyl	Herbicide
Bifenazate	Bifenazate	Acaracide
Bifenthrin	Bifenthrin	Insecticide
Bromacil	Bromacil	Herbicide
Bromophos-Ethyl	Bromophos-Ethyl	Insecticide
Bromopropylate	Bromopropylate	Acaricide
Bromoxynil	Bromoxynil	Herbicide
Boscalid	Boscalid	Fungicide
Bupirimate	Bupirimate	Fungicide
Butachlor	Butachlor	Herbicide
Butralin	Butralin	Herbicide
Butylate	Butylate	Herbicide
Captan	Captan	Fungicide
Carbaryl	Carbaryl	Insecticide
Carbofuran	Carbofuran	Insecticide
Carfentrazone-ethyl	Carfentrazone-ethyl	Herbicide
cis-Chlordane	cis-Chlordane	Insecticide
t-Chlordane	t-Chlordane	Insecticide
Chlormephos	Chlormephos	Insecticide
Chloroneb	Chloroneb	Fungicide
Chlorothalonil	Chlorothalonil	Fungicide
Chlorpyrifos	Chlorpyrifos	Insecticide
Chlorpyrifos-Methyl	Chlorpyrifos-Methyl	Insecticide
Chlorthal-Dimethyl	Chlorthal-Dimethyl	Insecticide
Chlorthiamid	Chlorthiamid	Herbicide

Clodinafop-propargyl	Clodinafop-propargyl	Herbicide
Clomazone	Clomazone	Herbicide
Clopyralid	Clopyralid	Herbicide
Cycloate	Cycloate	Herbicide
Cyfluthrin	Cyfluthrin	Insecticide
Cypermethrin-beta	Cypermethrin-beta	Insecticide
Cypermethrin-zeta	Cypermethrin-zeta	Insecticide
Cyhalothrin lambda	Cyhalothrin lambda	Insecticide
Cyprodinil	Cyprodinil	Fungicide
o,p-Dichlorodiphenyldichloroethane	o,p-Dichlorodiphenyldichloroethane	Insecticide
p,p-Dichlorodiphenyldichloroethane	p,p-Dichlorodiphenyldichloroethane	Insecticide
o,p-Dichlorodiphenyldichloroethylene	o,p-Dichlorodiphenyldichloroethylene	Insecticide
p,p-Dichlorodiphenyldichloroethylene	p,p-Dichlorodiphenyldichloroethylene	Insecticide
o,p-Dichlorodiphenyltrichloroethane	o,p-Dichlorodiphenyltrichloroethane	Insecticide
p,p-Dichlorodiphenyltrichloroethane	p,p-Dichlorodiphenyltrichloroethane	Insecticide
Deltamethrin	Deltamethrin	Insecticide
Desmetryn	Desmetryn	Herbicide
Diazinon	Diazinon	Insecticide
Dicamba	Dicamba	Herbicide
Dichlobenil	Dichlobenil	Herbicide
Dichlorprop	Dichlorprop	Herbicide
Dichlorvos	Dichlorvos	Insecticide
Dichlofenthion	Dichlofenthion	Nematicide
Diclofop	Diclofop	Herbicide
Dieldrin	Dieldrin	Insecticide
Difenoconazole	Difenoconazole	Fungicide
Dimethachlor	Dimethachlor	Herbicide
Dimethoate	Dimethoate	Insecticide
Dioxathion	Dioxathion	Insecticide
Diphenamid	Diphenamid	Herbicide
α-Endosulfan	α-Endosulfan	Insecticide
Endrin	Endrin	Insecticide
S-ethyl dipropylthiocarbamate	S-ethyl dipropylthiocarbamate	Herbicide
Ethalfluralin	Ethalfluralin	Herbicide
Ethion	Ethion	Insecticide
Ethofumesate	Ethofumesate	Herbicide
Etradiazole	Etradiazole	Fungicide
Etrimphos	Etrimphos	Insecticide
Famoxadone	Famoxadone	Fungicide
Fenamidone	Fenamidone	Fungicide
Fenchlorphos	Fenchlorphos	Insecticide

Fenoxaprop	Fenoxaprop	Herbicide
Fenthion	Fenthion	Insecticide
Flamprop-Isopropyl	Flamprop-Isopropyl	Herbicide
Flamprop-Methyl	Flamprop-Methyl	Herbicide
Fluazifop-p-butyl	Fluazifop-p-butyl	Herbicide
Fludioxonil	Fludioxonil	Fungicide
Flumetralin	Flumetralin	Growth Regulator
Flumioxazin	Flumioxazin	Herbicide
Fluroxypyr	Fluroxypyr	Herbicide
Folpet	Folpet	Fungicide
Fonofos	Fonofos	Insecticide
Hexachlorocyclohexane-alpha	Hexachlorocyclohexane-alpha	Insecticide
Hexachlorocyclohexane-beta	Hexachlorocyclohexane-beta	Insecticide
Hexachlorocyclohexane-delta	Hexachlorocyclohexane-delta	Insecticide
Lindane (Hexachlorocyclohexane-gamma)	Lindane (Hexachlorocyclohexane-gamma)	Insecticide
Heptachlor	Heptachlor	Insecticide
tr-Heptachlor Epoxide	tr-Heptachlor Epoxide	Insecticide
Hexazinone	Hexazinone	Herbicide
Imazamethabenz	Imazamethabenz	Herbicide
Imazethapyr	Imazethapyr	Herbicide
Ipconazole	Ipconazole	Seed Treatment
Iprodione	Iprodione	Fungicide
Isofenphos	Isofenphos	Insecticide
Malathion	Malathion	Insecticide
MCPA (2-methyl-4-chlorophenoxyacetic acid)	MCPA (2-methyl-4-chlorophenoxyacetic acid)	Herbicide
MCPA-EHE	MCPA-EHE	Herbicide
MCPB-methyl	MCPB-methyl	Herbicide
Mecoprop (methylchlorophenoxypropionic acid)	Mecoprop (methylchlorophenoxypropionic acid)	Herbicide
Metalaxyl	Metalaxyl	Fungicide
Metconazole	Metconazole	Fungicide
Methoprene	Methoprene	Insecticide
Methoxychlor	Methoxychlor	Insecticide
Metolachlor	Metolachlor	Herbicide
Mirex	Mirex	Insecticide
Monolinuron	Monolinuron	Herbicide
Myclobutanil	Myclobutanil	Fungicide
Naled	Naled	Insecticide
Napropamide	Napropamide	Herbicide
Nitrapyrin	Nitrapyrin	Bactericide
Oxyfluorfen	Oxyfluorfen	Herbicide

Pendimethalin	Pendimethalin	Herbicide
cis-Permethrin	cis-Permethrin	Insecticide
trans-Permethrin	trans-Permethrin	Insecticide
Phorate	Phorate	Insecticide
Picloram	Picloram	Herbicide
Picoxystrobin	Picoxystrobin	Fungicide
Piperonyl butoxide	Piperonyl butoxide	Insecticide Synergist
Pirimicarb	Pirimicarb	Insecticide
Pirimiphos-Ethyl	Pirimiphos-Ethyl	Insecticide
Pirimiphos-Methyl	Pirimiphos-Methyl	Insecticide
Procymidone	Procymidone	Fungicide
Prometon	Prometon	Herbicide
Prometryn	Prometryn	Herbicide
Propetamphos	Propetamphos	Insecticide
Propham	Propham	Herbicide
Propiconazole	Propiconazole	Fungicide
Propoxur	Propoxur	Insecticide
Propyzamide	Propyzamide	Herbicide
Prothioconazole-Desthio	Prothioconazole-Desthio	Fungicide
Pyraclostrobin	Pyraclostrobin	Fungicide
Pyridaben	Pyridaben	Acaracide/insecticide
Pyrimethanil	Pyrimethanil	Fungicide/seed treat
Quinclorac	Quinclorac	Herbicide
Quintozene	Quintozene	Fungicide
Quizalofop-ethyl	Quizalofop-ethyl	Herbicide
Simazine	Simazine	Herbicide
Spiromesifen	Spiromesifen	Insecticide
Sulfentrazone	Sulfentrazone	Herbicide
Sulfotep	Sulfotep	Insecticide
Sulprophos	Sulprophos	Insecticide
Tebuconazole	Tebuconazole	Fungicide
Terbacil	Terbacil	Herbicide
Terbufos	Terbufos	Insecticide
Terbutryn	Terbutryn	Herbicide
Tetradifon	Tetradifon	Acaricide
Tetramethrin I	Tetramethrin I	Insecticide
Tetrasul	Tetrasul	Acaricide
Triallate	Triallate	Herbicide
Triclopyr	Triclopyr	Herbicide
Trifloxystrobin	Trifloxystrobin	Fungicide
Trifluralin	Trifluralin	Herbicide
Triticonazole	Triticonazole	Fungicide
Vinclozolin	Vinclozolin	Fungicide

Zoxamide	Zoxamide	Fungicide
	Chlorantraniliprole	Diamide
	Cyantraniliprole	Diamide
	Flonicamid	Insecticide
	Flubendiamide	Diamide
	Flupyradifurone	Insecticide
	Sulfoxaflor	Insecticide

Table A-6. Kruskal-Wallis test results for differences among sites for all non-factor environmental variables. Significant *p*-values are in bold and results of post-hoc Dunn's tests are in Tables A-9 and A-10.

	Year	χ^2	df	*P*
TDS	2018	23.571	4	**< 0.001**
Turbidity	2017	2.818	4	0.589
	2018	14.147	4	**0.007**
DN	2017	5.106	4	0.277
DP	2018	13.687	4	**0.008**
DNAmm	2017	14.259	4	**0.007**
	2018	4.523	4	0.340
TN	2017	6.548	4	0.162
	2018	18.391	4	**0.001**
TP	2018	10.732	4	**0.030**
PestDet	2017	12.268	4	**0.015**
	2018	13.914	4	**0.008**
PestSumConc	2017	17.917	4	**0.001**
	2018	14.318	4	**0.006**
RoadDist	2017	6.539	4	0.162
	2018	6.023	4	0.197
VegBuffWidth	2018	17.621	4	**0.001**
AlgaeCover	2017	1.279	4	0.865
	2018	2.656	4	0.617
PropWater	2017	22.754	4	**< 0.001**
	2018	37.319	4	**< 0.001**
PropExposed	2017	20.082	4	**< 0.001**
	2018	33.907	4	**< 0.001**
PropUrban	2017	6.628	4	0.157
	2018	17.336	4	**0.002**
PropNatural	2017	18.821	4	**0.001**
	2018	33.875	4	**< 0.001**
PropPasture	2017	18.468	4	**0.001**
	2018	32.801	4	**< 0.001**
PropCrops	2017	23.974	4	**< 0.001**
	2018	39.610	4	**< 0.001**
CPUE	2017	3.792	4	0.435
	2018	4.507	4	0.342

Table A-7. ANOVA test results for differences among sites for non-factor environmental variables. Significant *p*-values are in bold and results of post-hoc Tukey HSD tests are in Tables A-9 and A-10. * indicates that variable was log-transformed to meet assumption of normality. ** indicates that variable was transformed by $\log_{10}(X + 1)$ to meet assumption of normality.

	Year	*F*	df	*p*
DO	2017	0.666	4	0.623
	2018	5.260	4	**0.002**
pH	2017	3.249	4	**0.032**
	2018	10.173	4	< **0.001**
TDS*	2017	5.154	4	**0.005**
Chla*	2017	4.363	4	**0.010**
	2018	3.417	4	**0.017**
DN*	2018	2.574	4	0.052
DP*	2017	1.390	4	0.272
TP	2017	1.645	4	0.200
Surface Area*	2017	2.038	4	0.126
	2018	3.882	4	**0.009**
VegBuffWidth**	2017	2.491	4	0.074

Table A-8. Fisher's exact tests results for differences among sites for factor environmental variables. Significant *p*-values are in bold and results of post-hoc pairwise Fisher's exact tests are in Table A-11.

Variable	Year	*p*
Connectivity	2017	0.174
	2018	0.227
FillCode	2017	0.448
	2018	**0.027**
Situation	2017	0.096
	2018	**0.002**
VegCover	2017	0.056
	2018	< **0.001**
Classification	2017	0.889
	2018	0.446

Table A-9. Results of Dunn's tests (*Z*-values and *p*-values) and Tukey HSD tests (difference and *p*-values) to identify site-specific differences of environmental variables for 2017. Statistically significant *p*-values are in bold.

pH								
	Allan		St. Denis		Burr		Colonsay	
	Difference	*p*	Difference	*p*	Difference	*p*	Difference	*p*
Allan								
St. Denis	-0.243	0.925						
Burr	0.623	0.233	-0.865	**0.031**				
Colonsay	0.353	0.738	-0.595	0.218	-0.270	0.830		
Humboldt	0.470	0.533	-0.712	0.123	-0.153	0.979	0.117	0.992
Total Dissolved Solids								
	Allan		St. Denis		Burr		Colonsay	
	Difference	*p*	Difference	*p*	Difference	*p*	Difference	*p*
Allan								
St. Denis	-0.145	0.860						
Burr	-0.566	**0.006**	0.421	**0.035**				
Colonsay	-0.284	0.303	0.138	0.836	0.282	0.213		
Humboldt	-0.114	0.936	-0.031	0.999	0.452	**0.021**	0.170	0.711
Chlorophyll a								
	Allan		St. Denis		Burr		Colonsay	
	Difference	*p*	Difference	*p*	Difference	*p*	Difference	*p*
Allan								
St. Denis	-0.182	0.973						
Burr	0.118	0.994	-0.300	0.806				
Colonsay	-0.679	0.174	0.497	0.388	-0.797	**0.042**		
Humboldt	-0.793	0.102	0.612	0.239	-0.912	**0.023**	-0.114	0.993
Ammonia								
	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*P*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	-2.140	0.226						
Burr	1.123	1.000	-3.568	**0.004**				
Colonsay	-0.853	0.788	-1.462	0.863	-2.209	0.217		
Humboldt	-1.087	0.831	-1.117	1.000	-2.402	0.147	-0.295	0.768
Proportion Pesticides Detected								
	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	-0.456	0.648						
Burr	-1.672	0.567	1.290	0.789				
Colonsay	-2.888	**0.039**	2.580	0.089	-1.290	0.986		

Humboldt	-2.280	0.181	1.935	0.371	-0.645	1.000	0.645	1.000
Pesticide Sum Concentration								
	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	-0.331	0.741						
Burr	-1.099	1.000	0.815	1.000				
Colonsay	-3.276	**0.011**	3.124	**0.016**	-2.309	0.126		
Humboldt	-2.721	0.052	2.535	0.079	-1.720	0.427	0.589	1.000
Proportion Water								
	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	0.468	0.640						
Burr	3.950	**0.001**	-3.692	**0.002**				
Colonsay	2.734	0.050	-2.397	0.099	-1.359	0.697		
Humboldt	1.442	0.746	-1.034	0.603	-2.613	0.063	-1.317	0.563
Proportion Exposed								
	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	1.609	0.538						
Burr	2.280	0.158	-0.648	1.000				
Colonsay	-0.017	0.987	1.801	0.431	-2.568	0.082		
Humboldt	-1.434	0.455	3.227	**0.011**	-4.019	**0.001**	-1.570	0.466
Proportion Natural								
	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	0.000	1.000						
Burr	3.106	**0.017**	-3.311	**0.009**				
Colonsay	1.958	0.251	-2.087	0.221	-1.283	0.798		
Humboldt	2.768	**0.040**	-2.935	**0.027**	-0.245	1.000	0.979	0.983
Proportion Pasture								
	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	0.882	1.000						
Burr	3.260	**0.010**	-2.498	0.075				
Colonsay	2.819	**0.039**	-2.027	0.213	-0.494	1.000		
Humboldt	3.314	**0.009**	-2.579	0.069	0.195	0.845	0.666	1.000
Proportion Crops								

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	-0.877	0.380						
Burr	-4.254	**0.000**	3.563	**0.003**				
Colonsay	-3.038	**0.019**	2.267	0.140	1.359	0.697		
Humboldt	-1.852	0.320	1.034	0.603	2.483	0.091	1.188	0.705

Table A-10. Results of Dunn's tests (*Z*-values and *p*-values) and Tukey HSD tests (difference and *p*-values) to identify site-specific differences of environmental variables for 2018. Statistically significant *p*-values are in bold.

DO

	Allan		St. Denis		Burr		Colonsay	
	Difference	*p*	Difference	*p*	Difference	*p*	Difference	*p*
Allan								
St. Denis	0.740	0.982						
Burr	3.133	0.182	-2.392	0.298				
Colonsay	3.042	0.189	-2.302	0.309	-0.090	1.000		
Humboldt	5.187	**0.004**	-4.447	**0.005**	2.055	0.449	2.145	0.378

pH

	Allan		St. Denis		Burr		Colonsay	
	Difference	*p*	Difference	*p*	Difference	*p*	Difference	*p*
Allan								
St. Denis	-0.324	0.727						
Burr	0.213	0.929	-0.537	0.162				
Colonsay	0.437	0.460	-0.761	**0.014**	0.224	0.868		
Humboldt	1.054	**0.002**	-1.378	**0.000**	0.841	**0.007**	0.617	0.067

Total Dissolved Solids

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	1.843	0.327						
Burr	3.692	**0.002**	-2.163	0.214				
Colonsay	0.767	0.887	1.243	0.856	-3.373	**0.006**		
Humboldt	-0.029	0.976	2.162	0.184	-4.268	**0.000**	-0.919	1.000

Turbidity

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	2.346	0.152						
Burr	3.036	**0.022**	-0.846	1.000				
Colonsay	1.977	0.336	0.426	0.670	-1.260	0.830		
Humboldt	3.511	**0.004**	-1.346	0.892	0.464	1.000	1.772	0.459

Chlorophyll a

	Allan		St. Denis		Burr		Colonsay	
	Difference	*p*	Difference	*p*	Difference	*p*	Difference	*p*
Allan								
St. Denis	-0.176	0.956						
Burr	-0.424	0.478	0.248	0.803				
Colonsay	-0.748	**0.039**	0.572	0.086	-0.324	0.604		

Humboldt	-0.654	0.092	0.478	0.206	-0.230	0.844	0.095	0.992

Dissolved Phosphorus

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	-2.569	**0.082**						
Burr	-3.046	**0.023**	0.606	1.000				
Colonsay	-2.832	**0.042**	0.362	1.000	0.239	0.811		
Humboldt	-1.212	0.903	-1.567	0.585	2.132	0.231	1.887	0.355

Total Nitrogen

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	-1.632	0.616						
Burr	1.477	0.699	-3.528	**0.004**				
Colonsay	-1.823	0.478	0.221	0.825	-3.743	**0.002**		
Humboldt	-0.998	1.000	-0.732	0.928	-2.815	**0.039**	0.953	1.000

Total Phosphorus

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	-1.926	0.325						
Burr	-2.562	0.104	0.814	1.000				
Colonsay	-2.530	0.103	0.740	0.919	0.094	0.925		
Humboldt	-0.888	1.000	-1.271	1.000	2.051	0.322	2.011	0.311

Proportion Pesticides Detected

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	-0.045	1.000						
Burr	-2.499	0.112	2.603	0.092				
Colonsay	-2.184	0.145	2.269	0.140	0.334	1.000		
Humboldt	-2.376	0.122	2.460	0.111	-0.006	0.996	-0.321	1.000

Pesticide Sum Concentration

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	-0.077	0.939						
Burr	-2.055	0.199	2.098	0.215				
Colonsay	-2.714	0.060	2.798	0.051	-0.699	1.000		
Humboldt	-2.346	0.133	2.396	0.133	-0.418	1.000	0.242	1.000

Surface Area

	Allan		St. Denis		Burr		Colonsay	
	Difference	*p*	Difference	*p*	Difference	*p*	Difference	*p*
Allan								
St. Denis	-1.109	**0.008**						
Burr	-0.555	0.416	-0.553	0.285				
Colonsay	-0.540	0.423	-0.568	0.236	0.015	1.000		
Humboldt	-0.942	**0.033**	-0.167	0.971	-0.386	0.634	-0.401	0.574

Vegetation Buffer Width

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	-3.170	**0.012**						
Burr	-4.021	**0.001**	1.050	1.000				
Colonsay	-3.310	**0.008**	0.162	0.872	0.892	1.000		
Humboldt	-2.765	**0.040**	-0.468	1.000	1.505	0.793	0.630	1.000

Proportion Water

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	0.374	0.709						
Burr	4.815	**0.000**	-5.104	**0.000**				
Colonsay	3.263	**0.008**	-3.337	**0.007**	-1.856	0.190		
Humboldt	2.113	0.173	-2.009	0.178	-3.148	**0.010**	-1.328	0.368

Proportion Exposed

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	2.298	0.129						
Burr	3.351	**0.006**	-1.261	0.622				
Colonsay	0.630	0.529	1.926	0.216	-3.136	**0.012**		
Humboldt	-1.245	0.426	4.091	**0.000**	-5.242	**0.000**	-2.165	0.152

Proportion Urban

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	2.165	0.213						
Burr	1.421	0.777	0.804	0.843				
Colonsay	-1.145	0.756	3.822	**0.001**	-2.917	**0.032**		
Humboldt	0.219	0.827	2.247	0.197	-1.384	0.666	1.575	0.692

Proportion Natural

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*

Allan								
St. Denis	0.079	0.937						
Burr	4.053	**0.000**	-4.561	**0.000**				
Colonsay	2.482	0.065	-2.775	**0.033**	-1.860	0.252		
Humboldt	3.558	**0.003**	-4.018	**0.000**	-0.650	1.000	1.243	0.642

Proportion Pasture

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*p*
Allan								
St. Denis	1.198	0.924						
Burr	4.205	**0.000**	-3.477	**0.004**				
Colonsay	3.930	**0.001**	-3.155	**0.008**	-0.407	1.000		
Humboldt	4.147	**0.000**	-3.405	**0.004**	-0.163	0.871	0.251	1.000

Proportion Crops

	Allan		St. Denis		Burr		Colonsay	
	Z	*p*	*Z*	*p*	*Z*	*p*	*Z*	*P*
Allan								
St. Denis	-1.180	0.476						
Burr	-5.385	**0.000**	4.852	**0.000**				
Colonsay	-3.775	**0.001**	2.996	**0.016**	1.935	0.159		
Humboldt	-3.037	**0.017**	2.145	0.128	2.764	**0.029**	0.851	0.395

Table A-11. Results of post-hoc pairwise Fisher's exact tests for factor environmental variables to identify site-specific differences of environmental variables for 2018. Statistically significant *p*-values are in bold.

Fill Code				
	Allan	St. Denis	Burr	Colonsay
	p	*p*	*p*	*p*
Allan				
St. Denis	0.237			
Burr	0.280	1		
Colonsay	1	0.237	0.311	
Humboldt	1	1	1	1
Situation				
	Allan	St. Denis	Burr	Colonsay
	p	*p*	*p*	*p*
Allan				
St. Denis	0.196			
Burr	1	0.920		
Colonsay	1	**0.007**	0.228	
Humboldt	0.762	0.920	1	**0.028**
VegCover				
	Allan	St. Denis	Burr	Colonsay
	p	*p*	*p*	*p*
Allan				
St. Denis	0.164			
Burr	0.054	1		
Colonsay	**0.014**	0.325	0.149	
Humboldt	0.584	0.682	0.441	0.584

Table A-12. Detection of ranavirus using eDNA over time through three sampling periods, summer 2017, spring 2018, and summer 2018. Blanks indicate wetlands that weren't sampled (2017) or had dried up (summer 2018).

Wetland	Summer 2017	Spring 2018	Summer 2018
Allan 1	-	-	+
Allan 2	-	-	+
Allan 3	-	-	+
Allan 4	+	-	-
Allan 8		-	-
Allan 10		-	-
Burr 1	-	-	-
Burr 2	-	-	-
Burr 5	-	-	-
Burr 6	-	-	-
Burr 9		-	-
Burr 10		-	-
Burr 11		-	-
Burr 12		-	
Burr 17		-	
Burr R1S	-	-	-
Burr R2N	-	+	-
Colonsay 1	-	-	-
Colonsay 2	-	-	-
Colonsay 3	-	-	-
Colonsay 4	-	-	+
Colonsay 5	-	-	-
Colonsay 6	-	-	+
Colonsay 7		-	-
Colonsay 8		-	+
Colonsay 9		-	+
Colonsay 10		-	-
Colonsay 11		-	
Colonsay 12		-	+
Colonsay 13		-	-
Colonsay 14		-	+
Colonsay 16		-	+
Colonsay 17		-	-
Colonsay 19		-	-
Colonsay 20		+	+
Humboldt 1	-	-	+
Humboldt 2	-	-	+

Humboldt 3	-	-	+
Humboldt 4	-	-	-
Humboldt 5	-	-	-
Humboldt 6		-	+
Humboldt 7		-	+
Humboldt 8		-	
Humboldt 13		-	-
Humboldt 18		-	-
Humboldt 19		-	-
Humboldt 20		-	+
Humboldt 21		-	-
Humboldt 22		-	-
Humboldt 24		+	+
Humboldt 25		-	
Humboldt 27		-	-
Humboldt 28		-	+
Humboldt 33		-	
Humboldt 36		-	-
Humboldt 41		-	-
Humboldt 100		-	-
St.Denis 1	-	-	-
St.Denis 2	+	-	-
St.Denis 3	+	-	+
St.Denis 4	+	-	+
St.Denis 5	-	-	+
St.Denis 6		-	-
St.Denis 7		-	+
St.Denis 8		-	-
St.Denis 9		-	+
St.Denis 12		-	-
St.Denis 18		-	-
St.Denis 19		-	+
St.Denis 20		-	+
St.Denis 23		-	-

Table A-13. Detection of wood frog presence or absence using eDNA compared to traditional detection methods through three sampling periods, summer 2017, spring 2018, and summer 2018. Blanks indicate wetlands that weren't sampled (2017) or had dried up (summer 2018). Traditional detections for summer 2018 are an accumulation and include those made in spring 2018.

Wetland	Summer 17		Spring 18		Summer 18	
	Traditional Detection	eDNA Detection	Traditional Detection	eDNA Detection	Traditional Detection	eDNA Detection
Allan 1	+	-	-	-	-	-
Allan 2	-	-	-	-	-	+
Allan 3	+	-	+	+	+	+
Allan 4	+	-	-	-	-	+
Allan 8			-	+	+	-
Allan 10			-	-	-	+
Burr 1	-	+	-	-	-	+
Burr 2	-	-	-	-	-	-
Burr 5	+	+	+	+	+	+
Burr 6	+	+	+	-	+	+
Burr 9			+	-	+	+
Burr 10			-	+	-	+
Burr 11			-	+	+	+
Burr 12			-	+		
Burr 17			-	-		
Burr R1S	-	-	-	-	-	-
Burr R2N	-	-	-	-	-	+
Colonsay 1	-	+	-	-	-	-
Colonsay 2	+	-	-	+	+	+
Colonsay 3	-	+	+	-	+	+
Colonsay 4	-	+	-	+	+	+
Colonsay 5	+	+	+	+	+	+
Colonsay 6	+	+	-	+	+	+
Colonsay 7			-	+	-	-
Colonsay 8			-	-	-	-
Colonsay 9			-	-	-	+
Colonsay 10			-	-	-	-
Colonsay 11			-	-		
Colonsay 12			-	+	-	+
Colonsay 13			+	+	+	+
Colonsay 14			-	-	-	+
Colonsay 16			+	+	+	+
Colonsay 17			-	+	-	-

Colonsay 19			-	+	-	+
Colonsay 20			+	+	+	+
Humboldt 1	-	-	-	-	-	+
Humboldt 2	-	+	-	-	-	+
Humboldt 3	-	-	-	-	-	-
Humboldt 4	-	+	-	-	-	-
Humboldt 5	-	-	-	-	-	+
Humboldt 6			-	+	-	+
Humboldt 7			-	+	-	+
Humboldt 8			-	-		
Humboldt 13			-	-	-	-
Humboldt 18			-	+	-	-
Humboldt 19			-	-	-	-
Humboldt 20			-	-	-	-
Humboldt 21			-	-	-	+
Humboldt 22			-	+	-	+
Humboldt 24			-	-	-	+
Humboldt 25			-	-		
Humboldt 27			-	-	-	+
Humboldt 28			-	+	-	+
Humboldt 33			-	+		
Humboldt 36			-	-	-	-
Humboldt 41			-	-	+	+
Humboldt 100			-	-	-	-
St.Denis 1	+	+	-	-	-	-
St.Denis 2	-	+	-	-	+	+
St.Denis 3	+	-	-	-	-	+
St.Denis 4	+	+	-	-	-	+
St.Denis 5	-	+	+	+	+	+
St.Denis 6			+	-	+	+
St.Denis 7			-	-	-	+
St.Denis 8			-	-	-	+
St.Denis 9			-	-	-	+
St.Denis 12			-	+	-	+
St.Denis 18			-	-	-	+
St.Denis 19			-	-	-	+
St.Denis 20			-	-	-	-
St.Denis 23			-	-	-	-

Table A-14. Relative influence values of predictor variables in the BRT model containing all variables (WF.tc3.lr0005.bf6), and in that containing only those that performed better than random (WF.tc3.lr0005.bf6.simp). See Table A-1 for variable descriptions.

	WF.tc3.lr0005.bf6		WF.tc3.lr0005.bf6.simp	
Rank	Variable	Relative Influence	Variable	Relative Influence
1	DP	21.95	DP	22.16
2	DN	11.80	DN	12.84
3	PropWater	8.16	PropWater	9.44
4	Chla	8.06	Chla	8.65
5	TDS	6.97	TDS	7.58
6	CPUE	6.85	CPUE	7.05
7	VegBuffWidth	4.49	VegBuffWidth	5.11
8	PropExposed	3.92	PropExposed	3.86
9	pH	3.01	pH	3.34
10	PropUrban	2.64	TN	3.03
11	TN	2.55	PropPasture	3.01
12	PropPasture	2.36	RoadDist	2.97
13	RoadDist	1.99	PropUrban	2.95
14	DNAmm	1.92	DNAmm	2.69
15	PropNatural	1.80	PropNatural	2.19
16	PropCrops	1.51	PropCrops	1.66
17	DO	1.39	DO	1.46
18	RandNum	1.31		
19	SurfaceArea	1.30		
20	Turbidity	1.01		
21	SurroundingLand	1.00		
22	PestSumConc	0.98		
23	VegCover	0.69		
24	AlgaeCover	0.52		
25	Classification	0.40		
26	Year	0.39		
27	Situation	0.35		
28	FillCode	0.24		
29	RVeDNA	0.19		
30	PestDet	0.19		
31	Connectivity	0.05		
32	Bovid	0.00		
33	Fish	0.00		

Table A-15. Tally of collected tadpoles (n), metamorphs (n), and blood smears (slides) for each wetland and site in 2017.

Site	Wetland	Type	n	Slides
St. Denis	1	tadpole	10	10
		metamorph	0	0
Burr	5	tadpole	24	15
		metamorph	0	0
	6	tadpole	15	13
		metamorph	0	0
Colonsay	5	tadpole	51	18
		metamorph	7	7
	6	tadpole	21	15
		metamorph	0	0

Table A-16. Tally of collected tadpoles (n), metamorphs (n), and blood smears (slides) for each wetland and site in 2018.

Site	Wetland	Type	N	Slides
Allan	3	tadpole	10	10
		metamorph	16	16
St. Denis	2	tadpole	4	4
		metamorph	2	2
	5	tadpole	15	15
		metamorph	15	14
	6	tadpole	16	12
		metamorph	0	0
Burr	5	tadpole	18	16
		metamorph	14	13
	11	tadpole	14	14
		metamorph	9	8
Colonsay	5	tadpole	16	10
		metamorph	15	15
	6	tadpole	17	15
		metamorph	0	0
	16	tadpole	15	12
		metamorph	15	15

Table A-17. Mean, median, standard deviation, minimum and maximum values of tadpole mass, body condition (k), and blood N:L ratios for each site.

Year	Site		Mass (g)	k	N:L
2017	St. Denis	Mean	1.0	0.022	0.499
		Median	1.0	0.021	0.404
		SD	0.4	0.004	0.441
		Min	0.4	0.015	0.082
		Max	1.6	0.030	1.500
	Burr	Mean	2.5	0.017	0.654
		Median	2.5	0.017	0.323
		SD	0.2	0.003	0.876
		Min	2.1	0.012	0.092
		Max	2.8	0.021	3.789
	Colonsay	Mean	0.7	0.019	0.463
		Median	0.6	0.015	0.235
		SD	0.5	0.023	0.749
		Min	0.1	0.007	0.043
		Max	1.8	0.160	4.143
2018	Allan	Mean	2.5	0.014	0.194
		Median	2.7	0.014	0.156
		SD	0.6	0.001	0.117
		Min	1.1	0.013	0.081
		Max	3.2	0.016	0.417
	St. Denis	Mean	2.1	0.015	0.192
		Median	2.2	0.015	0.159
		SD	1.0	0.002	0.115
		Min	0.4	0.010	0.034
		Max	3.9	0.020	0.447
	Burr	Mean	1.8	0.017	1.306
		Median	1.9	0.017	0.337
		SD	0.5	0.003	1.990
		Min	0.7	0.013	0.038
		Max	2.6	0.024	8.000
	Colonsay	Mean	1.5	0.017	0.308
		Median	1.0	0.017	0.222
		SD	1.2	0.003	0.316
		Min	0.2	0.009	0.056
		Max	4.0	0.030	1.667

Table A-18. Mean, median, standard deviation, minimum and maximum values of metamorph mass, body condition (k), and blood N:L ratios for each site.

Year	Site		Mass (g)	k	N:L
2017	Colonsay	Mean	0.8	0.014	0.116
		Median	0.8	0.014	0.103
		SD	0.2	0.004	0.044
		Min	0.5	0.009	0.071
		Max	1.0	0.020	0.208
2018	Allan	Mean	2.1	0.016	0.104
		Median	2.1	0.016	0.078
		SD	0.3	0.002	0.076
		Min	1.2	0.013	0.000
		Max	2.6	0.019	0.288
	St. Denis	Mean	1.7	0.011	0.128
		Median	1.6	0.011	0.100
		SD	0.3	0.002	0.090
		Min	1.3	0.008	0.023
		Max	2.3	0.015	0.393
	Burr	Mean	1.4	0.011	0.323
		Median	1.4	0.010	0.179
		SD	0.2	0.003	0.384
		Min	1.0	0.008	0.000
		Max	2.0	0.019	1.433
	Colonsay	Mean	1.3	0.011	0.287
		Median	1.3	0.010	0.204
		SD	0.2	0.003	0.327
		Min	0.9	0.007	0.056
		Max	1.7	0.019	1.619

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