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Author

Ruso, GE, Hogan, NS, Sheedy, C, Gallant, MJ, Jardine, TD

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Effects of Agricultural Stressors on Growth and an Immune Status Indicator in Wood Frog (*Lithobates sylvaticus*) Tadpoles and Metamorphs

Gabrielle E. Ruso,[a,*] Natacha S. Hogan,[b,c] Claudia Sheedy,[d,1] Melanie J. Gallant,[a] and Timothy D. Jardine[b,e]

[a]Toxicology Graduate Program, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[b]Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[c]Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[d]Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, Lethbridge, Alberta, Canada
[e]School of Environment and Sustainability, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Abstract: Like many amphibians, wood frog (*Lithobates sylvaticus*) populations have likely declined or experienced local extirpations as a result of habitat alterations. Despite this, wood frogs are still present and breeding in altered landscapes, like the agricultural Prairie Pothole Region of central Canada, and are exposed to a variety of anthropogenic impacts. As tadpoles, water contamination can have negative effects on growth, development, and immune systems. To investigate the potential effects of agricultural land use on tadpole growth and immune system stress, we used boosted regression trees to model body mass, body condition, and neutrophil to lymphocyte ratios, a measure of immune stress, against 32 variables including water quality, wetland habitat, and landscape-level measures. Developmental stage strongly influenced all 3 endpoints, and body mass was negatively influenced by higher levels of total dissolved solids (>600–700 mg/L) and at the first sign of pesticide detection (>0.01 proportion pesticides detected of those screened). While correlative, these data suggest that tadpoles developing in agricultural environments may experience survival and reproductive disadvantages if they metamorphose at smaller body sizes. Given the potential impacts this can have on adult frogs and frog populations, these results provide an impetus for further field-based investigation into the effects that pesticides, and especially total dissolved solids, may have on tadpoles. *Environ Toxicol Chem* 2021;40:2269–2281. © 2021 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Agriculture; Amphibians; Immune system; Pesticides; Prairie Pothole Region; Wetlands

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 as a result of 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 semipermanent 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 affect population growth rates are influential during the larval and juvenile stages and include factors such as predation, food

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[1]Deceased.
* Address correspondence to ruso.gaby2@gmail.com
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quantity, water temperature, and the likelihood of the water body to desiccate prematurely (Semlitsch and Bridges 2005). The addition of anthropogenic habitat alteration and climate change puts greater stress on this life stage, when successful metamorphosis is critical to adult survival and fecundity (Relyea 2004; Smith et al. 2011; Todd 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 also been identified as a leading threat to 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 (Lesbarrères et al. 2014).

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) as well as 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. There is evidence to suggest that laboratory-based experiments may overestimate effects of contaminants compared to field studies (Lanctôt et al. 2014), but various mesocosm studies illustrate the potential for real-world impacts. Agricultural contaminants can also have indirect effects on amphibians by altering habitat and predator–prey interactions (Gibbons et al. 2015). For example, the broadleaf herbicide atrazine can cause reductions in periphyton abundance and 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 cause reduced tadpole growth and development (Relyea and Diecks 2008). In another study, however, atrazine has also been shown to indirectly enhance 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, effecting 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 are critical for the individual's 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; Peace et al. 2019). 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 infections 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, though increasing, investigations (Davis 2009; Shutler and Marcogliese 2011). Nevertheless, recent research suggests that pesticide exposure can influence immune system function (see Kiesecker 2002; Johnson and Sutherland 2003; Pochini and Hoverman 2017). For example, 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 median lethal concentration (LC50) value of those pesticides. Given the growing concern of ranavirus-related die-offs of amphibian populations, it is important to consider the multiple, interacting stressors that affect tadpole immune system health, including the presence of ranavirus, contaminants, and overall habitat degradation (Robert 2010).

In light of this knowledge, we investigated the potential effects of agricultural land use on tadpole and metamorph size and development and N:L ratios in a field setting. Across 5 sites that vary in agricultural intensity (from grasslands and tamed pastures to croplands), we surveyed for tadpoles and collected data on morphometrics and blood immune cell ratios. We used boosted regression tree (BRT) models to evaluate how a variety of water quality, wetland habitat, and landscape-level variables affected tadpole growth and immune system stress. We predicted that individuals from the agriculture-intensive (cropland) sites would show reduced growth and stressed immune systems as evidenced by elevated N:L ratios.

METHODS

Study area and wetland selection

We targeted tadpole collection from a total of 26 wetlands at 5 sites between May and July of 2017 and 71 wetlands at the same sites between May and July of 2018. We chose these sites for their accessibility and previous use in other studies (Stanton et al. 2016; Michelson et al. 2018; also see Ruso et al. 2019). The sites are divided into 2 grassland (including tamed pasture) sites—Allan, SK (51.6260 N, −105.9717 W), and St. Denis National Wildlife Area (52.2153 N, −106.0770 W); and 3 cropland sites—Burr, SK (51.9809 N, −105.0774 W), Colonsay, SK (52.0264 N, −105.9217 W), and Humboldt, SK (52.1993 N, −105.2883 W). In 2017, we found few wetlands containing tadpoles, so in 2018, we increased the total number of surveyed wetlands. To do this, we established circular survey zones that still contained the original wetlands surveyed in 2017 but expanded the surveyed area, and thus the number of wetlands, in a standardized manner. We created single circles with radii of 0.4 km or 2 circles with radii of 0.28 km each, depending on how the originally surveyed wetlands were spatially distributed. Within these circles, we used satellite imagery and ground-truthing to survey all of the "new" wetlands. In 2017,

we performed dipnet surveys to look for tadpoles at all wetlands, but in 2018, we only performed dipnet surveys at the 33 wetlands where we saw egg masses or where we saw or heard adult wood frogs. Of the 26 wetlands surveyed in 2017, 5 contained tadpoles, and of the 71 surveyed in 2018, 12 contained tadpoles; but only 9 wetlands had enough tadpoles for collection (i.e., more than one or 2 tadpoles caught per hour of effort; Table 1). Wood frog tadpoles were typically the only species identified in these wetlands (Preston 1982). At the smallest and largest wetlands within each site, we placed temperature loggers (Onset HOBO 8 K pendant, UA-001-08) <1 m below the surface of the water to record temperature once every hour and retrieved the loggers when we completed fieldwork. In 2017, we were able to place a pair of loggers at each site, totaling 10 loggers; but in 2018, we were only able to place pairs at St. Denis, Burr, and Colonsay, totaling 6 loggers, because of limited supply. We placed loggers at the smallest and largest wetlands to assess general differences between the expected extreme ends of wetland temperature profiles at each site. 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 that there were no large differences among sites that could confound comparisons; but we did not have sufficient data to include temperature in the models. Additional details and temperature data can be found in Supplemental Data, Figures S1 and S2.

Water quality, wetland habitat, and landscape-level variables

For modeling purposes, we only used tadpoles and metamorphs that had associated habitat data (see below, *Body condition and body mass*). The variables measured in these models are the same as those reported previously (Ruso et al. 2019) and are briefly described in the present study (see Supplemental Data, Table S1, for additional details and methods). For water quality, we collected data on dissolved oxygen (milligrams per liter), pH, total dissolved solids (milligrams per liter), turbidity (formazin nephelometric units), chlorophyll a (micrograms per liter), dissolved nitrogen (nitrate, milligrams per liter of N, and ammonia, milligrams per liter of N), dissolved phosphorus (phosphate, milligrams per liter of P), total nitrogen (milligrams per liter of N), and pesticide detections and concentrations (Supplemental Data, Table S2). Although we collected data on total phosphorus (milligrams per liter of P) and conductivity (microsiemens per centimeter), they were highly correlated with dissolved phosphorus and total dissolved solids, respectively, and thus removed from the models. Pesticide detection was calculated as the proportion of pesticides detected out of the total number screened for (166 in 2017 and 172 in 2018), and pesticide concentrations were measured as the sum concentration of all pesticides. In this case, using the sum concentration of all pesticides, including herbicides, insecticides, and fungicides, violates the assumptions that all of the detected pesticides act through similar modes of action and have equal toxicity. We opted to use this method, however, because there were very low concentrations of any one pesticide, we could not find LC50 or median effect concentration values for every detected pesticide for wood frogs, and we did not want to make assumptions about toxicity across taxa. For wetland habitat, we collected data on surface area (square meters), fish presence or absence, connectivity to other wetlands, fill status, surrounding land use or crop type, distance to the nearest road (meters), situation (hydrogeomorphology), vegetation cover type, vegetation buffer width (meters), percentage of algae cover, and wetland classification (i.e., permanency). For landscape-level features, we used ArcGIS (Environmental Systems Resource Institute 2017) and the raster data land-use file for 2016 produced by Agriculture and Agri-Food Canada (2016) to calculate the proportional area of surrounding land within a 1-km-radius circle from the center of each wetland as either crops (barley + oats + spring wheat + canola and rapeseed + lentils + soybeans + peas), pasture and forage, natural (coniferous + broadleaf + mixed wood forests + shrubland + grassland and prairies), urban and developed (including roads), exposed and barren, or water and wetlands.

In addition to these wetland variables, we measured catch-per-unit-effort, a measure of tadpole density, and detection of ranavirus. Catch-per-unit-effort was measured as the number of tadpoles caught per dipnet sweep. Ranavirus was recorded as presence or absence as indicated by environmental DNA

TABLE 1: Number of wetlands that were surveyed for tadpoles, number where tadpoles and metamorphs were found and collected, and number of wetlands for which tadpole and metamorph data were used in the boosted regression tree models at 5 study sites in Saskatchewan, Canada, in 2017 and 2018

		2017			2018		
Location	Type	Wetlands surveyed	Wetlands with tadpoles	Wetlands used in models	Wetlands surveyed	Wetlands with tadpoles	Wetlands used in models
Allan	Grassland	4	0	0	6	1	1
St. Denis	Grassland	5	1	1	14	3	3
Burr	Cropland	6	2	2	11	2	2
Colonsay[a]	Cropland	6	2	2	18	3	1
Humboldt	Cropland	5	0	0	22	0	0

[a]Note that there is a difference between the number of wetlands with tadpoles and the number of wetlands included in the models for Colonsay in 2018 because only a subset of the wetlands had all of the water quality and wetland habitat measurements collected (see *Methods* for further details).

(eDNA) testing (Picco et al. 2007; also see Supplemental Data, Table S1, and Ruso et al. [2019] for additional details).

Tadpole and metamorph collection and processing

Tadpoles were collected using dipnets, aiming for approximately 15 individuals per wetland (similar to Forbes et al. 2006; Schutler and Marcogliese 2011; Sifkarovski et al. 2014), and we recorded body mass (0.1 g), snout–vent length (SVL, mm), and Gosner stage (Gosner 1960). We calculated body condition as mass divided by SVL^3, then multiplied by 100 (Lanctôt et al. 2014). Tadpoles and metamorphs were euthanized by an overdose of tricaine mesylate. We 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, we collected metamorphic frogs (Gosner stage ≥42) with dipnets or by hand and processed them similarly, again aiming for 15 individuals per wetland. We recorded mass, SVL, and Gosner stage; collected blood samples for blood smears; and preserved all tissues. When the tail was almost or completely absorbed in metamorphs or when we could not get sufficient volume from the tail, we took blood from the heart. If we caught more than 15 tadpoles or metamorphs during the dipnet sweeps, then the remaining individuals were released to their wetland of origin. We made blood smears in the field by smearing the blood on a microscope slide and allowing it to air-dry before being stored and transported back to the laboratory. In the laboratory, we stained the slides with Protocol Hema 3 stain (Fisher Scientific) and preserved them with mounted coverslips. To prevent cross-contamination, all dissections and blood smear processes were performed with clean gloves and tools. To reduce observer bias, one individual (G.E. Ruso) performed all microscopy and counted all white blood cells in a back-and-forth, zigzag pattern up to 100 under a microscope at ×1000 magnification by oil immersion and calculated N:L ratios. All samples were collected under a Saskatchewan Ministry of Environment research permit (no. 17FW204), an Environment and Climate Change Canada National Wildlife Area access permit (no. 2017-072), and with University of Saskatchewan Animal Use Protocol approval (no. 20170055).

Statistical analyses

To assess the effect of the 32 explanatory variables related to water quality (including pesticide detection and sum concentration), wetland habitat, and land use and cover (Supplemental Data, Table S1) on tadpole morphology (body condition [*k*] and body mass) and blood N:L ratios, we used BRT models. In addition to the wetland variables, we included year, catch-per-unit-effort, ranavirus presence or absence, and Gosner stage, to account for temporal developmental effects, as explanatory variables. We did not include wetland temperatures in the models because few wetlands were monitored; 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 was greater than random (Soykan et al. 2014; Main et al. 2015). To improve normality of the response variables, we log-transformed body condition, and for N:L ratios we log-transformed by $\log_{10}(X+0.1)$. Body mass did not require transformation. We selected BRT models over linear mixed effects models because BRTs are more lenient toward data sets with variables of multiple scales, collinearity, and missing data, while maintaining interpretability (De'ath 2007; Elith et al. 2008). Briefly, a BRT model combines thousands of regression trees to first describe general trends in the data and then the remaining variation (Elith et al. 2008). The BRT model parameters include tree complexity (i.e., the number of nodes, or decision points, in each tree), bag fraction (the proportion of data used to create each tree or "step"), and learning rate (the weight given to each tree in a complete BRT model; Elith et al. 2008). We kept tree complexity at 3 for both models because of low sample size (i.e., number of wetlands sampled) and difficulty in describing greater than 3-way interactions (Main et al. 2015) but used exploratory analyses to determine optimal values for the bag fraction and learning rate. To identify the optimal BRT models, we examined percentage of cross-validated deviance explained. Because the response variables were continuous, not binomial, we could not use a measure of area under the receiver operating characteristic curve as an indicator of model performance. To interpret model results, we focused on the relative influence values of each variable and the partial dependence plots (De'ath 2007; Elith et al. 2008; Soykan et al. 2014; Main et al. 2015). The relative influence value is a measure of how useful a variable is and is determined by how many times it is included in a tree and how much it improves the overall BRT model (reduced deviance; Elith et al. 2008). These relative influence values are scaled such that the sum of the values for all model variables equals 100 (Elith et al. 2008). Partial dependence plots show the relationship between the response variable and a single explanatory variable while holding the effects of all other explanatory variables at average (De'ath 2007; Elith et al. 2008). Summary statistics were calculated in Microsoft Excel, and all additional analyses were conducted in R, Ver 3.5.1 (R Development Core Team 2018).

RESULTS

Wood frog tadpole, metamorph, and blood smear collections

In 2017, we collected tadpoles, metamorphs, and blood smears between 3 June and 21 July. In total, we collected 121 tadpoles and 7 metamorphs (Table 2). In 2018, we collected tadpoles, metamorphs, and blood smears between 5 June and 5 July and collected a total of 125 tadpoles and 86 metamorphs (Table 3). In both years there were cases in which wetlands desiccated before tadpoles could metamorphose, resulting in fewer metamorphs collected than tadpoles. Blood

TABLE 2: Tally of collected tadpoles (*n*), metamorphs (*n*), and blood smears (slides) for each wetland and site in 2017 in Saskatchewan, Canada

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

smears were collected from as many tadpoles and metamorphs as possible, although there were individuals for which we could not get enough blood or it was too watery (Tables 2 and 3). In 2017, we collected a total of 78 blood smears and in 2018, a total of 191.

Without yet accounting for Gosner stage, overall mean and median tadpole body mass appeared to vary strongly across sites in 2017, with the lowest mean and median values of 0.7 and 0.6 g, respectively, at Colonsay, a cropland site, and the highest values being 2.5 g at Burr, also a cropland site (Table 4). Of the metamorphs collected at Colonsay, the mean and median values were 0.8 g (Table 5). Summarizing 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 (Figure 1). The range of Gosner stages collected in 2017 at Burr was 25 to 41, at Colonsay this range was 21 to 45, and at St. Denis it was 33 to 38. We 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 throughout their development at each site. However, our ability to collect tadpoles throughout their development was hindered by the order in which we found tadpole-containing wetlands within each site. At certain sites, we did not find tadpoles until later in their development because of the randomness with which we visited and surveyed wetlands. In addition, the tadpoles collected at St. Denis in 2017 were from a rapidly desiccating wetland.

TABLE 3: Tally of collected tadpoles (*n*), metamorphs (*n*), and blood smears (slides) for each wetland and site in 2018 in Saskatchewan, Canada

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 4: Mean, median, standard deviation, and minimum and maximum values of tadpole mass, body condition, and blood neutrophil to lymphocyte ratios for each site in Saskatchewan, Canada

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

k = body condition; N:L = neutrophil to lymphocyte; SD = standard deviation.

In 2018, there was less variation in mean and median mass across sites for both tadpoles and metamorphs (Tables 4 and 5). Mean and median tadpole mass ranged from 1.5 to 2.5 and from 1.0 to 2.7 g, respectively. Mean and median metamorph mass both ranged from 1.3 to 2.1 g. The range of Gosner stages collected at each site was 34 to 44 at Allan, 31 to 46 at St. Denis, 32 to 46 at Burr, and 27 to 46 at Colonsay. As in 2017, we purposefully rotated site visitation throughout each week to try to capture tadpoles throughout their development, but the effectiveness of this was limited by the random order in which we visited wetlands and likely by wetland-specific differences in temperature regimes (Supplemental Data, Figures S1 and S2).

TABLE 5: Mean, median, standard deviation, and minimum and maximum values of metamorph mass, body condition, and blood neutrophil to lymphocyte ratios for each site in Saskatchewan, Canada

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

k = body condition; N:L = neutrophil to lymphocyte; SD = standard deviation.

When SVL was taken into account by calculating *k*, differences among sites were minimal in both years for tadpoles and metamorphs (Tables 4 and 5). 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 <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. Out of the 269 total blood smears collected, 25 N:L ratios were >1. In both years at Burr, in particular, tadpoles and metamorphs exhibited a higher mean and a wider range of N:L ratio values (Tables 4 and 5). This can be at least partially explained by an abundance of unique neutrophils (Figure 2). Unique neutrophils were identified as neutrophils with tiny, dark pink granules in the cytoplasm. These granules tended to be very small (i.e., "pinprick") but appeared to occasionally clump together and look larger and irregularly shaped (Figure 2). The granules were usually sparsely distributed throughout the cytoplasm but sometimes were also fairly dense.

Modeling tadpole growth and health

Initial runs of BRT models for *k*, 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 because exploratory analyses showed that increasing this value did not improve model performance, indicated by the percentage of cross-validated deviance explained (Table 6). The percentages of cross-validated deviance explained by the *k* and mass BRT models were much greater than that of the N:L model (Table 6). For the *k* and N:L models, only Gosner stage performed better than a random number (Supplemental Data, Table S3). Body condition declined with increasing Gosner stage but especially so at metamorphic climax (~Gosner stage 41–42), and N:L ratio was generally stable until metamorphic climax, at which point it also sharply declined.

The initial BRT model for body mass indicated that 5 variables had a relative influence greater than random, including Gosner stage, total dissolved solids (milligrams per liter), proportion of pesticides detected, ammonia (NH_3-N milligrams per liter), and wetland surface area (square meters; Supplemental Data, Table S3). When a simplified mass model was run, the percentage of cross-validated deviance explained increased slightly by 2% (Table 6). The partial dependence plots illustrate the influence of these 5 variables on tadpole and metamorph mass (Figure 3). Mass increased with Gosner stage until metamorphic climax and then declined (Figure 3A). There is an apparent threshold for total dissolved solids of approximately 600 to 700 mg/L, above which there is a negative effect on mass (Figure 3B). At seemingly the first sign of pesticide detection (0.01, i.e., 1%) there is also a negative influence on mass, but as the proportion of pesticides detected increased, the negative influence increased quite slowly (Figure 3C). 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 (Figure 3D). Similar to ammonia, surface area has a generally neutral effect on mass except for a slight positive influence when it is between 4000 and 6000 m^2 (Figure 3E). Using mass as a response variable indicated that certain water quality variables are influential, but when SVL is accounted for by using *k* as the response, these variables appear to have no influence (Supplemental Data, Table S3).

DISCUSSION

General findings

The results of the present study provide evidence for successful wood frog breeding at wetlands in both grassland and cropland sites. The BRT models indicate that, aside from the effects of Gosner stage, there were no environmental influences on *k* or N:L ratios. Conversely, body mass alone 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 total dissolved solids and pesticides showed defined, negative effects. We also observed broad differences in wetland temperature regimes, generally based on wetland size, and found some smaller wetlands with tadpoles desiccating before metamorphosis could be completed. In several of the desiccating wetlands, we observed tadpoles "stress-morphing." In response to stressors, such as pond desiccation, tadpoles may

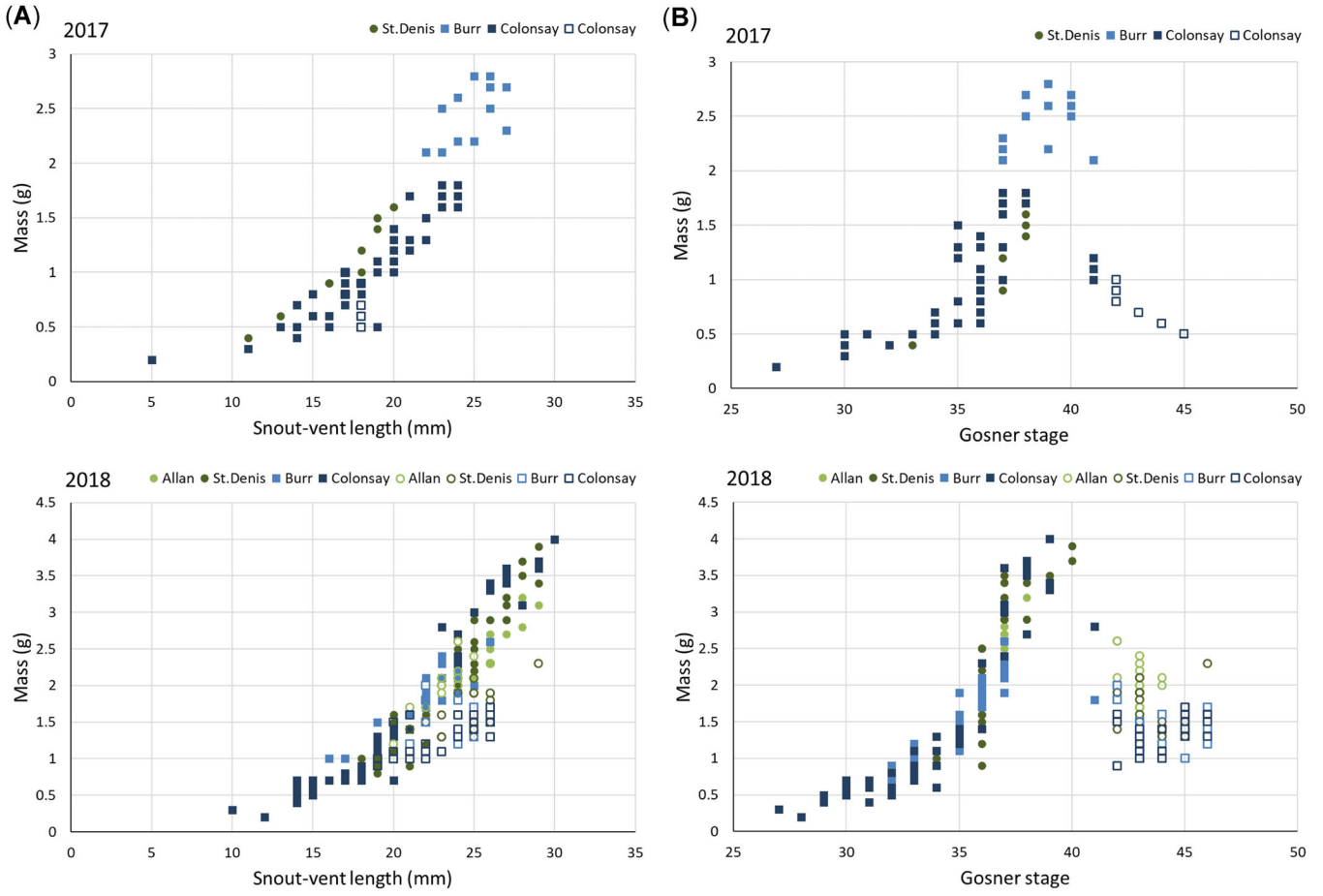

FIGURE 1: Relationships between mass and snout–vent length (**A**) and between mass and Gosner stage (**B**) of wood frog tadpoles and metamorphs collected at 4 sites in 2017 and 2018 in Saskatchewan, Canada. Circles represent grassland sites (Allan, St. Denis), and squares represent cropland sites (Burr, Colonsay). Solid points represent tadpoles, and hollow points represent metamorphs.

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). Although 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

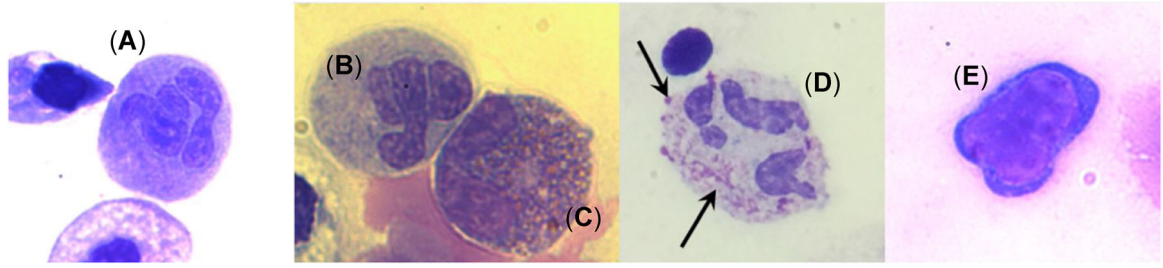

FIGURE 2: Examples of neutrophil and eosinophil white blood cells in the blood smears of wood frog tadpoles and metamorphs. Typical neutrophils (**A**, **B**) have segmented nuclei with lavender cytoplasm clear of any granules; eosinophils (**C**) have relatively large pink-magenta granules; unique neutrophils (**D**) have small, pinprick, dark pink granules typically distributed patchily throughout the cytoplasm; and lymphocytes (**E**) have purple nuclei and very little cytoplasm. When these unique neutrophils were found in a blood smear, they were often very abundant. All photographs were taken at ×1000 magnification by oil immersion.

TABLE 6: Parameters and evaluation metrices of the best-performing boosted regression tree (BRT) models for body condition, blood neutrophil to lymphocyte ratios, and the top full (all variables) and simplified (only variables better than random; M.tc3.lr005.bf5.simp) BRT models for mass

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	*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

lr = learning rate; *bf* = bag fraction; *tc* = tree complexity; *nt* = optimal number of trees; dev = deviance; CV = cross-validated; *k* = body condition; N:L = neutrophil to lymphocyte.

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, indicating that tadpoles in smaller ponds are likely exposed to greater high and low extremes. Studies with Alaskan wood frogs have shown that tadpole development can be influenced by small changes in temperature, although after hatching, tadpoles can move freely and may seek optimal temperatures such that the effect of temperature on post-hatching development may be somewhat diluted by other factors (Herreid and Kinney 1967). Further, development and growth rates are likely affected by other environmental factors in addition to water temperature, including density and photoperiod (Smith-Gill and Berven 1979; Laurila et al. 2001). In light of predicted effects of climate change on Prairie Pothole Region wetlands (e.g., warmer temperatures and more severe drought [Barnett et al. 2005]), tadpoles in smaller wetlands may be forced to develop faster in response to temperature increase and wetland desiccation. This 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, making broad, range-wide conclusions about the potential effect of wetland temperatures on wood frog tadpoles is difficult (Berven and Gill 1983).

Factors influencing tadpole and metamorph growth and health

Body condition and body mass. Initial modeling attempts used body condition as the response variable to test for potential effects of environmental variables on tadpole mass while accounting for SVL. However, the only variable that had a stronger influence than a random number was Gosner stage. Body

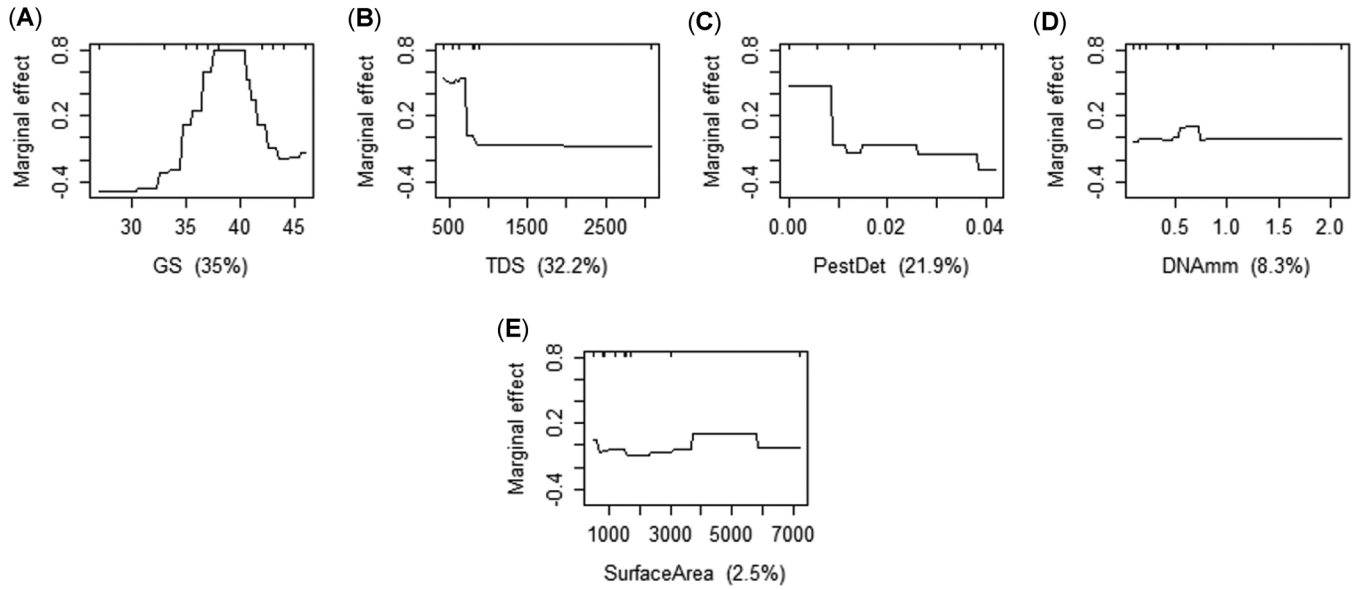

FIGURE 3: Partial dependence plots (**A**–**E**) for the simplified mass boosted regression tree model showing the 5 variables with relative influence greater than a random number: Gosner stage, total dissolved solids (milligrams per liter), proportion of pesticides detected, dissolved nitrogen–ammonia (milligrams per liter), and wetland surface area (see Supplemental Data, Table S1, for further detail). Marginal effect is the effect of the variable of interest on tadpole or metamorph mass (grams) 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). GS = Gosner stage; TDS = total dissolved solids; PestDet = proportion of pesticides detected; DNAmm = dissolved nitrogen–ammonia.

condition was clearly influenced by metamorphosis as it remained stable or slightly declined until metamorphic climax (Gosner stage 41–42), at which point it dropped precipitously. To test if there was any influence of additional variables on tadpole and metamorph growth alone, we ran BRT models using body mass as the response variable and found that although 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 random. The effect of Gosner stage on mass matched that of the raw data in that mass steadily increased until metamorphic climax when it 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 approximately 600 to 700 mg/L, above which there was a negative influence on body mass. Total dissolved solids are a known concern for tadpoles in the context of road salt chlorides and may have lethal and nonlethal effects, especially in chronic exposures (Sanzo and Hecnar 2006). Brine contamination from oil and gas exploration can also reduce amphibian abundance and survival (Hossack et al. 2017, 2018), but this is not of concern in this region. Rather, high-salinity wetlands in this region of Saskatchewan tend to have sulfate salts (Rawson and Moore 1944; Hammer 1978; Last and Ginn 2005), and few studies have been conducted using these as contaminants of concern. Elphick et al. (2011) studied the toxicity of sulfate on several freshwater organisms 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 (80–100 mg/L) and hard (160–250 mg/L) water, they proposed guidelines of 644 and 725 mg/L sulfate, respectively. These values are similar to the apparent total dissolved solids threshold for mass found in the present study, but they are not easily comparable given that the actual ion constituency and water hardness of the sampled wetlands are unknown.

The BRT model indicated that at approximately 0.01 proportion of pesticides detected within a wetland, there was a negative influence on body mass. Because 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 12 wetlands included in these models, 5 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 because there were detections in 2018; and we 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 2 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 discrepancy between the influence of pesticide detection and sum pesticide concentration may be due to small sample size (only 5 of the wetlands included in the models were tested and had detections), but the presence of a small effect also suggests that more intensive field-based studies may help us understand the level of impact that pesticides may have on tadpoles in real-world scenarios.

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). Johansson 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 an $LC50_{120h}$ 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 range substantially, from at least 8.05 to >270 mg/L (Vardia et al. 1984; Morgan 1996). Relyea (2005) also found no effect of 2,4-D on tadpoles. Each of these studies, however, was a single-contaminant test. 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 are the sole factor 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]) and 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 are somewhat difficult to interpret. Both variables had fairly neutral effects except when ammonia concentrations were approximately 0.5 to 0.75 NH_3-N mg/L (0.61–0.91 NH_3 mg/L) and when wetland surface area was approximately 4000 to 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 in the present study 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 effects of ammonia on body mass, based on the BRT

model, occur 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 the present 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 on body mass 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. 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. 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 *k* and body mass BRT models reflect the differences in each metric's implication. The *k* 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 simply assesses overall size because mass and SVL are linearly related. 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 *k*, and vice versa, depending on Gosner stage. There is some concern over using *k* to assess tadpole energy stores because of 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 may be more insightful (MacCracken and Stebbings 2012). Nevertheless, both of these metrics, *k* and body mass, are frequently applied in anuran research and provide interesting comparisons.

Both models in the present study are also limited by their data sets. Because of the haphazard nature with which we selected wetlands to use for water quality and wetland habitat measurements in 2018, we 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. We felt that this was too limited a data set for these individuals to warrant including them in the models. In some cases (e.g., wetland Colonsay6 in 2017), however, pesticide data were not collected, but all other variables were measured; and the associated tadpoles and metamorphs were kept in the BRT models. So, although there are many tadpole and metamorph individuals included in each model ($n = 292$), they are sourced only from 12 wetlands in total. Therefore, the individuals are not wholly independent of each other, and the explanatory data associated with those individuals are highly repetitive according to wetland origin, representing an important limitation of these results.

N:L ratios. Like the BRT model for *k*, 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 may be dictated simply by metamorphosis. The 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 appears 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 (Davis 2009). Overall, however, our 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). We 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). Little research has been done on amphibian white blood cells, especially with wood frog tadpoles; and we 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, we concluded that they were likely neutrophils with 1° granules (M. Forzán, Long Island University, New York, NY, USA, and M. Meachem, University of Saskatchewan, Saskatoon, SK, Canada, 2018, personal communication). Primary granules are generally thought of as storage sites of toxic mediators that may be released to kill bacteria or other pathogens (Lacy 2006). These findings may indicate some level of pathogenic stress in these tadpoles and metamorphs that may not have been accounted for by the measured explanatory variables, namely the presence of ranavirus. Ranavirus was detected at only 2 wetlands, neither of which were at Burr. Despite the potential for ranavirus infection to influence tadpole leukocyte profiles, the exact ways in which these profiles are affected may change over the course of infection and are also influenced by environmental factors (Forzán et al. 2016). The fact that we did not test each individual tadpole or metamorph for ranavirus infection and our use of field-based, rather than laboratory-reared, organisms may contribute to the lack of ranavirus effect in this BRT model.

Besides the anomalous neutrophils, another caveat of this data set, which may or may not have affected the outcome of the BRT, was related to the difficulty of collecting blood from tadpoles and metamorphs. With tadpoles, we could typically get enough blood from the tail, but there was frequently other fluid associated with these collections that may have introduced leukocytes circulating

from other parts of the tadpole body into the sample. It was also difficult to get blood from the tails of metamorphs, and we 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 *k* and mass, the percent deviance explained through cross-validated 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 (e.g., complete temperature data, measures of food quality or competition), or improved 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 laboratory settings to better control for confounding factors, including environmental variables and handling stress, and the potential to use flow cytometry, which may improve classification of the unique neutrophils.

CONCLUSIONS AND FURTHER IMPLICATIONS

Despite the somewhat limited data set and lack of environmental effects on either *k* or N:L ratios, we 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 total dissolved solids and proportion of pesticides detected are the only explanation for reduced mass, but they stand out as important factors and provide impetus for further laboratory- and 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 ion constituents of total dissolved solids on tadpoles, other than effects of seawater (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 Prairie Pothole Region (Ruso et al. 2019) will improve our understanding of their ecology and their susceptibility to additional natural and anthropogenic stressors (Hopkins and Brodie 2015). Although effects observed in the present study were only found for mass, not *k* 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 all act on individuals simultaneously and may have effects that are different from those observed in laboratory-based investigations (Lanctôt et al. 2014).

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5107.

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Author Contributions Statement—G.E. Ruso designed the project, collected field and laboratory data, analyzed the data, and wrote the manuscript; N.S. Hogan provided guidance for project design, scientific advice regarding immune system questions, and comments and edits of the manuscript; C. Sheedy processed pesticide samples and provided comments and edits of the manuscript; M.J. Gallant cultured ranavirus for eDNA work and provided comments and edits of the manuscript; and T.D. Jardine provided guidance for project design, contributed to statistical analyses and interpretation, and provided comments and edits of the manuscript.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (ruso.gaby2@gmail.com).

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