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Leukocyte Profiles of Northern Leopard Frogs, *Lithobates pipiens*, Exposed to Pesticides and Hematozoa in Agricultural Wetlands

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Contaminants, including pesticides, can affect an organism's health by weakening its immune system, potentially making it more susceptible to parasites. We used one measure of immune function, leukocyte profiles, and compared these for 82 Northern Leopard Frogs, *Lithobates* (=*Rana*) *pipiens*, occupying five wetlands with different exposures to the herbicides atrazine and metolachlor, and tested for associations between blood parasites and leukocyte profiles. *Hepatozoon* spp. (likely *H. clamitae*) were the only blood parasites detected and were found in only seven (8.5%) frogs. These parasites were associated with fewer eosinophils per leukocyte and higher heterophil:lymphocyte ratios, although we cannot distinguish cause and effect. Heterophils, lymphocytes, and eosinophils per total number of leukocytes also differed significantly among wetlands, but variation could not be attributed consistently to pesticide contamination.

AMPHIBIANS occupying anthropogenically modified landscapes are subjected to novel challenges with which they have had little time to co-evolve. Among vertebrates, amphibians may be particularly susceptible to anthropogenic changes because they have experienced a comparatively higher rate of extinction in recent years (Houlahan et al., 2000; Stuart et al., 2004). Some changes, such as wetland drainage resulting in habitat loss, have clear and direct effects on amphibian populations. Other changes, such as contaminants, may have both lethal and sublethal effects. Amphibians are vulnerable to a variety of contaminants, including pesticides, heavy metals, and other pollutants. Contaminants can arise from various urban, industrial, and agricultural sources, and may reach high concentrations in wetlands commonly inhabited by amphibians. Contaminants, including pesticides, can weaken immune responses (Carey et al., 1999, 2003; Voccia et al., 1999), potentially leading to increased risk of parasitism and disease, further endangering populations in decline. In particular, amphibians are exposed to pesticides applied during aquatic larval phases of development and again as adults that congregate in wetlands to breed, both of which may affect immunocompetence (Carey et al., 1999). Natural stressors, such as parasites and pathogens, have also been implicated in amphibian declines (Stuart et al., 2004; Vredenburg et al., 2010). Herein we test the hypothesis that pesticide exposure, immune function, and blood parasitism are correlated.

Leukocyte ratios provide one measure of immune function (Norris and Evans, 2000). However, amphibian granulocytic leukocytes are relatively poorly studied compared to those of other vertebrates. They are described based on morphology as heterophilic granulocytes, eosinophilic granulocytes, basophilic granulocytes, and neutrophilic granulocytes (Wright, 2001). However, they are often referred to as heterophils, eosinophils, basophils, and neutrophils (Wright, 2001), and we follow this terminology. Heterophils are often synonymized with neutrophils in amphibians, but neutrophils (=neutrophilic granulocytes, see above) are morphologically distinct from heterophils (=heterophilic granulocytes) and are a relatively uncommon cell type (<5% of all leukocytes; Wright, 2001). Heterophils and monocytes are phagocytic and generally associated with investment in innate immunity, whereas lymphocytes, the smallest leukocyte, represent investment in acquired immunity. Eosinophils are cytotoxic cells that stimulate other white blood cells to release histamines and protect hosts from helminth parasites (Edwards, 1994). In birds and mammals, higher heterophil to lymphocyte ratios are indicative of various kinds of stressor, including fasting, foreign antigens (e.g., of parasites), pesticides, handling, blood-sampling, and injury (Gross and Siegel, 1983; Vleck et al., 2000; Work et al., 2001; Shutler et al., 2004). The same has been reported for heterophil:lymphocyte ratios in amphibians (referred to as neutrophil:lymphocyte ratios in Forbes et al., 2006; Davis et al., 2008), which is considered a general response to any stressor.

It is crucial to understand effects of agricultural practices on amphibian health because farm ponds provide important, and in some areas the only, habitat for breeding populations of frogs and salamanders (Knutson et al., 2004). In this study, we examine leukocyte profiles of Northern Leopard Frogs, *Lithobates* (=*Rana*) *pipiens* (hereafter frogs) occupying wetlands exposed to herbicides (atrazine and metolachlor) versus reference wetlands that do not receive direct herbicide input from adjacent agricultural activity. Exposure to pesticides, in particular atrazine, is associated with immunosuppression in amphibians (Carey and Bryant, 1995; Voccia et al., 1999; Rohr and McCoy, 2010). Atrazine and metolachlor rank second and tenth in herbicide sales in the United States (Lehman and Williams, 2010). In general, effects of exposure to metolachlor have been rarely studied in amphibians, but include DNA and thymus damage (Lehman and Williams, 2010).

We used blood smears to quantify hematozoan infections in frogs to test if they were associated with differences in leukocyte profiles because parasites can affect biomarkers of animal health, including differential white blood cell counts (Marcogliese et al., 2009). Whereas agricultural effects on macroparasites have been explored in several frog species, including Northern Leopard Frogs at the sites used in this study (King et al., 2007, 2008, 2010; Marcogliese et al.,

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Table 1. Descriptions of Study Sites from Which Northern Leopard Frogs Were Collected in September 2006. Pesticide data were collected in 2005 and are reported in King et al. (2007). Study sites are ranked in terms of increasing pesticide concentrations; the first three are reference sites and next two are contaminated sites. Additional site characteristics are provided in King et al. (2007).

Name	Short name in text	Coordinates	Pesticides[a] (Mean and range in μg/L)	*n* frogs
Étang John Sauro	Sauro	45°04′N, 73°09′W	Atrazine (0.02; 02–0.04) Metolachlor (0.03)	18
Parc le Rocher	Rocher	45°38′N, 73°19′W	Atrazine (0.02; 0.01–0.03) Metolachlor (0.03)	16
Île de la Commune	Commune	45°37′N, 73°28′W	Atrazine (0.04; 0.02–0.06) Metolachlor (0.06; 0.02–0.15)	18
Ruisseau Fairbanks	Fairbanks	45°01′N, 73°21′W	Atrazine (0.19; 0.03–0.35) Metolachlor (0.07; 0.03–0.14)	17
Rivière Chibouet	Chibouet	45°47′N, 72°49′W	Atrazine (0.75; 0.0–3.70) Metolachlor (0.33; 0.04–0.89)	13

[a] If no range is reported, readings were identical each time they were measured.

2009), protozoan parasites in the blood have never been examined in this context. However, infections with another microparasite, the chytrid fungus *Batrachochytrium dendrobatidis*, were associated with altered leukocyte profiles in Bullfrogs, *Lithobates* (=*Rana*) *catesbeianus* (Davis et al., 2010).

MATERIALS AND METHODS

Recently metamorphosed juvenile frogs were collected by hand or dip net from five wetlands in southwestern Quebec, Canada from 5–11 September 2006 (Table 1). Typically, metamorphosis occurs in mid-July in this region. Thus, all frogs collected for this study were approximately seven to eight weeks post-metamorphosis. Collecting this soon after metamorphosis minimized the likelihood that frogs had moved among wetlands (King et al., 2007, 2008). Two wetlands (Fairbanks and Chibouet) consistently have high levels of herbicides that are received from adjacent farmlands that annually apply pesticides (King et al., 2007, 2008). Pesticide levels at these sites are typically high, and at Chibouet they are above CCME (2010) guidelines for atrazine (1.8 μg/L; King et al., 2007, 2008). At Fairbanks, atrazine concentrations do not reach these levels, but they nevertheless are markedly higher than at Sauro, Rocher, and Commune. Atrazine is typically applied in June, when Northern Leopard Frog tadpoles are undergoing larval development, a period which may be sensitive to contaminants for future immunological and endocrinological function (Carey and Bryant, 1995). However, effects of exposure to pesticides, including atrazine, may last beyond the period of larval exposure (Lehman and Williams, 2010). Atrazine has a half-life ranging from less than a week to 7–8 months, and metolachlor, over six months (CCME, 2010). The other three wetlands do not receive direct influx of herbicides from agricultural activities and were considered reference sites. Concentrations of atrazine and metolachlor are well below those attained at the other two sites and well below CCME guidelines (King et al., 2007). Sauro is a conservation area managed by Ducks Unlimited. Rocher is a managed wetland situated in a municipal park. Commune is adjacent to farmland, but is located in a provincial park where pesticide use is now forbidden. Atrazine and metolachlor were found here at 8.3 and 13.5 μg/L, respectively in 2001 (King et al., 2008), but improved to 0.04 and 0.06 μg/L, respectively in 2005 after park regulations were enforced (King et al., 2007). Concentrations are slightly higher than at the other two reference sites, likely because this site is exposed to waters of the St. Lawrence River, where atrazine measured 0.05 μg/L in 2003–04 (Rondeau, 2005). Although we acknowledge that pesticide measurements were not taken concurrent with frog collections, concentrations generally follow a repeated annual pattern due to routine farming practices, being consistently high when measured at Fairbanks in 2001 and 2005 and Chibouet in 2001, 2003, and 2004 (Boily et al., 2005; King et al., 2007, 2008). Furthermore, in the Chibouet River, atrazine concentration has surpassed recommended levels in June or early July each year since at least 1996 (Giroux, 1999, 2004, 2010). Localities are described in more detail in King et al. (2007).

On the day of capture, the heart of each frog was punctured with a sterile 27-gauge needle and approximately 20–40 mL of blood were collected in a heparinized capillary tube before being smeared on a microscope slide (Bennett, 1970) and fixed in alcohol. Slides were subsequently fixed and stained with Protocol Hema 3 (Biochemical Sciences Inc., Swedesboro, NJ). Frogs were killed in an overdose of buffered MS 222, and the bodies and tissues were frozen for other companion studies. Procedures adhered to the guidelines of the Canadian Council on Animal Care (2003).

We examined each smear with a compound microscope at 1000× magnification and distinguished the first 100 white blood cells (heterophils, lymphocytes, eosinophils, basophils, and monocytes) encountered, estimated the number of erythrocytes per field, counted the number of fields that were needed to achieve 100 white blood cells, and concomitantly searched for and enumerated blood parasites (*Hepatozoon* spp., *Lankesterella* spp., and *Trypanosoma* spp.). Density of white blood cells was computed as the proportion of white blood cells per 1000 red blood cells. Ten randomly chosen slides were re-examined to test repeatability. We also computed heterophil:lymphocyte ratios as a measure of response to parasite and pesticide stressors (Forbes et al., 2006; Davis et al., 2008). Slides were read blind to contaminant conditions in the wetlands.

Statistical analyses were carried out in SAS Version 9.1 (SAS Institute, Cary, NC). We used General Linear Models (GLMs) to compare blood smear data between parasitized and unparasitized frogs and among wetlands. Although most variables did not have normal distributions (Shapiro-Wilk tests, $Ps < 0.05$), GLMs were sufficiently robust because Kruskal-Wallis tests gave comparable results; the latter are not reported. If a GLM comparing wetlands was significant,

Table 2. Comparison of Leukocyte Profiles of Northern Leopard Frogs in Which No *Hepatozoon* spp. Were Detected ($n = 75$) Versus in Those in Which *Hepatozoon* spp. Were Detected ($n = 7$), and of Northern Leopard Frogs ($n = 52$) Sampled from Three Reference Wetlands Versus Those ($n = 30$) That Came from Two Contaminated Wetlands.

	Median	Range	Median	Range
	without *Hepatozoon*		with *Hepatozoon*	
% Heterophils	38.2	0.0–80.0	58.4	22.5–77.5
% Lymphocytes	33.0	7.0–91.1	26.0	5.9–65.3
% Eosinophils	17.4	0.0–77.7	6.9	0.0–18.8
Heterophil:Lymphocyte ratio	1.1	0.0–6.7	2.4	0.3–13.2
Heterophils per 1000 erythrocytes	19.6	0.0–114.3	24.9	13.3–56.4
Lymphocytes per 1000 erythrocytes	17.9	2.8–117.9	19.1	3.6–38.7
Eosinophils per 1000 erythrocytes	9.1	0.0–85.1	4.2	0.0–8. 0
	Reference sites		Sites with pesticides	
% Heterophils	50.0	14.0–80.0	34.2	0.0–69.6
% Lymphocytes	22.7	5.9–65.6	37.9	9.0–91.1
% Eosinophils	11.9	0.0–39.8	19.5	0.0–77.7
Heterophil:Lymphocyte ratio	2.4	0.3–13.2	1.0	0.0–6.3
Heterophils per 1000 erythrocytes	25.9	4.5–71.4	17.8	0.0–114.3
Lymphocytes per 1000 erythrocytes	15.5	3.6–38.7	19.5	2.8–117.9
Eosinophils per 1000 erythrocytes	6.1	0.0–46.9	9.5	0.0–85.1

Tukey tests were used for pairwise comparisons. To determine whether the number of analyses could be reduced, we tested whether principal component analysis (PCA) on the correlation matrix of leukocyte variables could be used to reduce individual leukocyte variables into a smaller number of principal components (PCs) that captured the majority of variation in leukocyte profiles. We used the broken stick model (Jackson, 1993), wherein the first PC of leukocyte counts must explain more variation than would be expected by chance. Means are reported ±SD.

RESULTS

Overall for the 82 frogs captured, percent leukocytes were 38.7 ± 20.1 heterophils, 36.0 ± 20.0 lymphocytes, 18.6 ± 14.2 eosinophils, 5.9 ± 8.0 basophils, and 0.8 ± 1.5 monocytes. There was significant repeatability for heterophils (Pearson's r for first and second smear readings = 0.92, $P = 0.0002$), lymphocytes ($r = 0.63$, $P = 0.05$), and eosinophils ($r = 0.92$, $P = 0.0002$), but not for basophils ($r = 0.35$, $P = 0.32$) or monocytes ($r = 0.16$, $P = 0.67$). Thus basophils and monocytes were not analyzed further. The first PC of the remaining three leukocyte variables explained less variation (58%) than expected by chance (61%), indicating that each leukocyte variable was independent and should be analyzed separately.

The only parasites we detected were *Hepatozoon* spp. (likely *H. clamatae*, but not reliably distinguished from *H. catesbianae* based on morphology alone; Kim et al., 1998; Boulianne et al., 2007) and in only seven of 82 (8.5%) frogs (maximum intensity 27 parasites per 100 leukocytes). Six of these came from Fairbanks, a contaminated site, and one from Sauro, a reference site. There were no differences in percents of leukocytes that were heterophils ($R^2 = 0.03$, $F_{1,80} = 2.6$, $P = 0.11$) or lymphocytes ($R^2 = 0.06$, $F_{1,80} = 0.4$, $P = 0.51$) between frogs infected with *Hepatozoon* spp. versus those that were not. However, parasitized frogs had lower percents of eosinophils than did unparasitized frogs (Table 2; $R^2 = 0.06$, $F_{1,80} = 4.8$, $P = 0.03$). Moreover, average heterophil:lymphocyte ratios were 4.0 for parasitized frogs and 1.7 for unparasitized frogs ($R^2 = 0.09$, $F_{1,80} = 7.9$, $P = 0.006$), consistent with an association between immunity and parasitism. There were no differences in densities of heterophils (ANOVA $R^2 = 0.003$, $F_{1,80} = 0.3$, $P = 0.58$), lymphocytes ($R^2 = 0.005$, $F_{1,80} = 0.4$, $P = 0.53$), eosinophils ($R^2 = 0.03$, $F_{1,80} = 2.7$, $P = 0.10$), or white blood cells ($R^2 = 0.002$, $F_{1,80} = 0.2$, $P = 0.70$) between frogs infected with *Hepatozoon* spp. versus those that were not.

There was significant variation among sites in percent heterophils (Fig. 1; $R^2 = 0.24$, $F_{4,77} = 6.2$, $P = 0.0002$), lymphocytes (Fig. 1, $R^2 = 0.21$, $F_{4,77} = 5.2$, $P = 0.001$), and eosinophils (Fig. 1, $R^2 = 0.12$, $F_{4,77} = 2.6$, $P = 0.04$). Percent heterophils was significantly higher at Fairbanks compared to Sauro and Commune, and percent lymphocytes was significantly higher at Sauro compared to Rocher and Fairbanks. Although the GLM suggested significant variation among sites for eosinophils ($R^2 = 0.12$, $F_{4,77} = 2.6$, $P = 0.04$), no Tukey pairwise comparisons were significant (Fig. 1; all Ps > 0.05). Similarly, there was significant variation among sites in densities of heterophils (Fig. 2; $R^2 = 0.14$, $F_{4,77} = 3.1$, $P = 0.02$), lymphocytes, and eosinophils (Fig. 2; identical test statistics for both the latter: $R^2 = 0.26$, $F_{4,77} = 6.7$, $P = 0.0001$). Heterophil density was significantly higher in Fairbanks compared to Rocher, lymphocyte density was significantly higher at Commune than all other sites except Sauro and at Sauro compared to Rocher, and eosinophil density was higher at Commune than at all other sites. Heterophil:lymphocyte ratios (computationally, this is the identical test for densities) also varied significantly among sites ($R^2 = 0.25$, $F_{4,77} = 6.6$, $P = 0.0001$); Fairbanks had significantly higher ratios ($\bar{x} = 3.8$) than all sites, but Rocher ($\bar{x} = 2.2$); Chibouet ($\bar{x} = 1.7$), Commune ($\bar{x} = 1.2$), and Sauro ($\bar{x} = 0.7$) were not significantly different from each other or Rocher (Tukey tests). Overall, reference sites had lower percents of heterophils (Table 2; $R^2 = 0.15$, $F_{1,80} = 14.2$, $P = 0.003$), higher percents of lymphocytes ($R^2 = 0.06$,

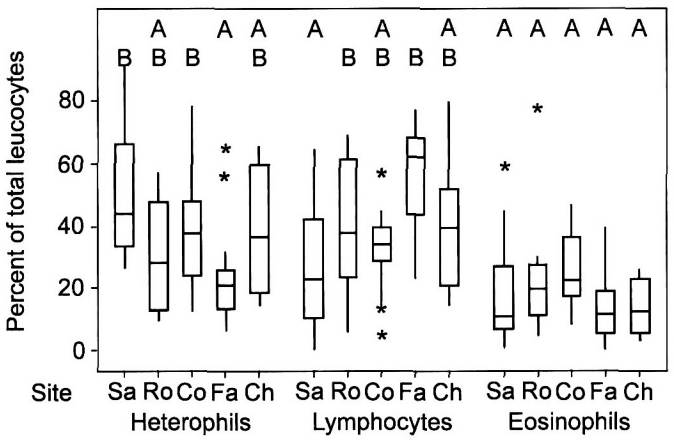

Fig. 1. Percent leukocytes from Northern Leopard Frogs (*Lithobates pipiens*) collected from five sites (Sa = Sauro, Ro = Rocher, Co = Commune, Fa = Fairbanks, Ch = Chibouet) in southwestern Quebec, Canada in September 2006. Sauro, Rocher, and Commune are reference sites (Table 1). Within blood cell types, sites sharing the same letter were not statistically different from each other at $P = 0.05$. Boxes show middle 50% of observations, horizontal line in box shows median, vertical lines show range of data excluding outliers (observations that are at least 1.5 times beyond the interquartile range), which are shown as asterisks. Sample sizes are provided in Table 1.

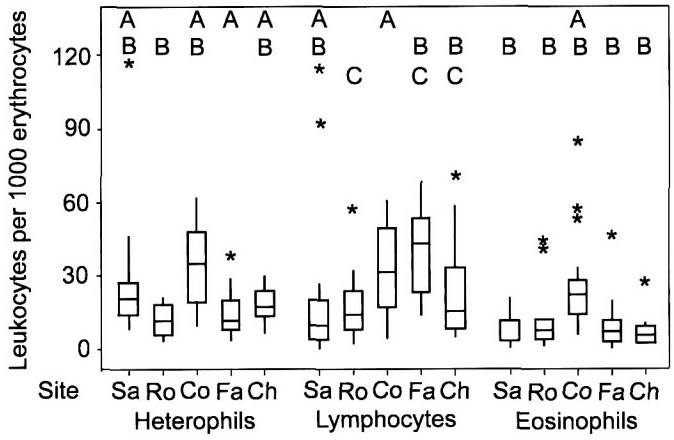

Fig. 2. Density (number of white blood cells per 1000 erythrocytes) of leukocytes from Northern Leopard Frogs (*Lithobates pipiens*) collected from five different sites. Site descriptions and symbols as in Fig. 1.

$F_{1,80} = 5.2$, $P = 0.03$) and eosinophils ($R^2 = 0.09$, $F_{1,80} = 7.6$, $P = 0.007$), and lower heterophil:lymphocyte ratios ($R^2 = 0.12$, $F_{1,80} = 11.0$, $P = 0.001$) than did sites receiving herbicides. Reference sites did not have significantly lower densities of heterophils (Table 2; $R^2 = 0.04$, $F_{1,80} = 3.2$, $P = 0.08$), but had higher densities of lymphocytes ($R^2 = 0.06$, $F_{1,80} = 5.3$, $P = 0.02$) and eosinophils ($R^2 = 0.05$, $F_{1,80} = 4.0$, $P = 0.05$) than did sites receiving pesticides.

DISCUSSION

Hepatozoon spp. are transmitted to frogs when they eat infected mosquitoes (Smith, 1996; Smith et al., 2000). As is the case for many haemosporidians in wild animals (e.g., Atkinson and van Riper, 1991; Desser and Bennett, 1993; Shutler et al., 1996), there is limited evidence of pathology (Kim et al., 1998; Smith et al., 2000). However, we found significant relationships between leukocytes and infection with *Hepatozoon* spp. Eosinophil proportions were lower and heterophil:lymphocyte ratios were higher in parasitized frogs, both of which may reflect a general stress response (Davis et al., 2008), or that may suggest that stress increased susceptibility to parasites. Generally, the clinical significance of intracellular blood parasites is unknown in amphibians (Allender and Fry, 2008). Other studies have shown that parasites can affect differential leukocyte profiles. Bullfrog tadpoles with clinical signs of chytridiomycosis had more neutrophils and fewer eosinophils than asymptomatic animals (Davis et al., 2010). Reduced eosinophilia associated with this fungal infection is similar to our observation of frogs infected with blood protozoans. In Bullfrogs, percent leukocytes, monocytes, and granulocytes were significantly affected by infection intensity of the lung trematode *Haematoloechus* sp. (Marcogliese et al., 2009). These results were context dependent in that effects of trematodes on different leukocyte components varied with atrazine concentrations. Infection of Northern Leopard Frogs by another lung parasite, the nematode *Rhabdias ranae*, following exposure to a pesticide mixture, also affected immune cell populations, enhancing lymphocyte proliferation and differentially affecting phagocytosis (Christin et al., 2003). In a separate study on Green Frogs, *Lithobates* (=*Rana*) *clamitans*, that were more frequently infected with *Hepatozoon* than our Northern Leopard Frogs, no relationships between parasitism and leukocyte profiles were observed (Shutler et al., 2009). These different results may reflect differences in parasite virulence among parasite–host systems or differences in pesticides or other stressors among study sites, suggesting that differences in immune function in response to blood parasites is context dependent.

We could not attribute differences in leukocyte profiles to herbicide exposure. On the one hand, frogs from the contaminated Fairbanks site had more heterophils, but Chibouet had the highest concentrations of atrazine and metolachlor, and frogs there had similar leukocyte profiles relative to other sites. However, differential leukocyte counts may reflect other aspects of habitat quality that were not measured in this study. For example, habitat characteristics such as exposure to cold temperatures can affect leukocyte counts (Maniero and Carey, 1997).

In fish, leukocyte profiles respond to water quality and appear to reflect immunocompetence (Hoole, 1997; Tierney et al., 2004), although various authors (Norris and Evans, 2000; Davis et al., 2008) have cautioned about inferring immune function from leukocyte profiles. Few studies of which we are aware have examined effects of pollution on leukocyte profiles in amphibians. Percent heterophils (neutrophils in their publication) decreased and eosinophils increased in *Rana* spp. from more polluted urban reservoirs compared to control sites (Romanova and Romanova, 2003). Shifts in proportions of leukocytes were also observed in *Pelophylax* (=*Rana*) spp. from a lake exposed to urban and agricultural runoff (Romanova and Egorikhina, 2006). However, proportions of eosinophils and monocytes were inconsistent, being high in some years and low in others. Numbers of heterophils (neutrophils in their publication) increased in Common Toads, *Chaunus* (=*Bufo*) *arenarum*, exposed to agricultural pesticides (Cabagna et al., 2005). In contrast, in another study on Bullfrogs, there was no effect of atrazine levels on differential leukocyte counts or percent leukocytes (Marcogliese et al., 2009). In a laboratory setting, exposure to

high concentrations of atrazine and nitrate each resulted in lower total leukocyte counts in the Tiger Salamander, *Ambystoma tigrinum* (Forson and Storfer, 2006). Eosinophil counts were reduced in Wood Frogs, *Lithobates* (=*Rana*) *sylvatica*, exposed to each of three different pesticides at most concentrations tested (Kiesecker, 2002) and in two species of *Lithobates* exposed to atrazine (Rohr et al., 2008). In Northern Leopard Frogs, lymphocyte proliferation was suppressed after exposure to a pesticide mixture containing atrazine (Christin et al., 2003). In contrast, atrazine exposure had no effect on haematocrit or hemoglobin concentration in *R. pipiens* (Allran and Karasov, 2000, 2001).

Our eosinophil counts are higher and monocyte counts lower than observed previously in amphibians in general (Davis and Durso, 2009:table 2; Shutler et al., 2009:table 1); remaining types of leukocytes fell within the range of two previous studies on Northern Leopard Frogs (Rouf, 1969; Maniero and Carey, 1997). The frogs used in this study were only seven to eight weeks post-metamorphosis and thus mostly younger than those used in the previous studies. It is not known if age affects leukocyte profiles in Northern Leopard Frogs, but heterophil counts increased with size in American Toads (Forbes et al., 2006), whereas no relationship between leukocyte profiles and size was observed in Northern Cricket Frogs, *Acris crepitans* (Davis and Durso, 2009). Eosinophils and monocytes reached maximum levels in developing Bullfrogs at metamorphosis and then decreased (Davis, 2009). Some of the variation in American Toad size in Forbes et al. (2006) may have been age related. The anatomical location from which blood is extracted may also affect leukocyte ratios; in particular, based on Shutler et al. (2009), in comparison to cardiac blood, peripheral blood appears to have more lymphocytes and fewer heterophils. However, because there are so few published data for amphibian leukocyte profiles and because profiles appear to change in response to a variety of environmental and biological factors (Foxon, 1964; Duellman and Trueb, 1986; Maniero and Carey, 1997; Davis, 2010), it is too early to make generalizations about how pesticides, parasites, and leukocyte profiles are related.

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LITERATURE CITED

Allender, M. C., and M. M. Fry. 2008. Amphibian hematology. Veterinary Clinics of North America: Exotic Animal Practice 11:463–480.

Allran, J. W., and W. H. Karasov. 2000. Effects of atrazine and nitrate on northern leopard frog (*Rana pipiens*) larvae exposed in the laboratory from posthatch through metamorphosis. Environmental Toxicology and Chemistry 19:2850–2855.

Allran, J. W., and W. H. Karasov. 2001. Effects of atrazine on embryos, larvae, and adults of anuran amphibians. Environmental Toxicology and Chemistry 20:769–775.

Atkinson, C. T., and C. van Riper, III. 1991. Pathogenicity and epizootiology of avian haematozoa: *Plasmodium*, *Leucocytozoon*, and *Haemoproteus*, p. 19–48. *In*: Bird–Parasite Interactions. J. E. Loye and M. Zuk (eds.). Oxford University Press, Oxford, U.K.

Bennett, G. F. 1970. Simple techniques for making avian blood smears. Canadian Journal of Zoology 48: 585–586.

Boily, M. H., V. F. Bérubé, P. A. Spear, C. DeBlois, and N. Dassylva. 2005. Hepatic retinoids of bullfrogs in relation to agricultural pesticides. Environmental Toxicology and Chemistry 24:1099–1106.

Boulianne, B. A. B., R. C. Evans, and T. G. Smith. 2007. Phylogenetic analysis of *Hepatozoon* species (Apicomplexa: Adeleorina) infecting frogs of Nova Scotia, Canada, determined by ITS-1 sequences. Journal of Parasitology 93:1435–1441.

Cabagna, M. C., R. C. Lajmanovich, G. Stringhini, J. C. Sanchez-Hernandez, and P. M. Pelzer. 2005. Hematological parameters of health status in the common toad *Bufo arenarum* in agroecosystems of Sante Fe Province, Argentina. Applied Herpetology 2:373–380.

Carey, C., D. F. Bradford, J. L. Brunner, J. P. Collins, E. W. Davidson, J. E. Longcore, M. Ouellet, A. P. Pessier, and D. M. Schock. 2003. Biotic factors in amphibian population declines, p. 153–208. *In*: Amphibian Decline: An Integrated Analysis of Multiple Stressor Effects. G. Linder, S. K. Krest, and D. W. Sparling (eds.). SETAC Press, Pensacola, Florida.

Carey, C., and C. J. Bryant. 1995. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environmental Health Perspectives 103(Supplement 4):13–17.

Carey, C., N. Cohen, and L. Rollins-Smith. 1999. Amphibian declines: an immunological perspective. Developmental and Comparative Immunology 23:459–472.

Canadian Council on Animal Care. 2003. Guidelines on: the care and use of wildlife. Available online: http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/GDLINES/Wildlife/Wildlife.pdf

CCME. 2010. Canadian Council of Ministers of the Environment. Canadian environmental quality guidelines summary table. http://st-ts.ccme.ca/?chems=10,136&chapters=1. Accessed 20 October 2010.

Christin, M. S., A. D. Gendron, P. Brousseau, L. Ménard, D. J. Marcogliese, D. Cyr, S. Ruby, and M. Fournier. 2003. Effects of agricultural pesticides on the immune system of *Rana pipiens* and on its resistance to parasitic infection. Environmental Toxicology and Chemistry 22:1127–1133.

Davis, A. K. 2009. The wildlife leukocytes webpage: the ecologist's source for information about leukocytes of wildlife species. www.wildlifehematology.uga.edu

Davis, A. K. 2010. Metamorphosis-related changes in leukocyte profiles of larval bullfrogs (*Rana catesbeiana*). Comparative and Clinical Pathology 18:181–186.

Davis, A. K., and A. M. Durso. 2009. White blood cell differentials of northern cricket frogs (*Acris c. crepitans*) with a compilation of published values from other amphibians. Herpetologica 65:260–267.

Davis, A. K., M. K. Keel, A. Ferreira, and J. C. Maerz. 2010. Effects of chytridiomycosis on circulating white blood cell distributions of bullfrog larvae (*Rana catesbeiana*). Comparative and Clinical Pathology 19:49–55.

Davis, A. K., D. L. Maney, and J. C. Maerz. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Functional Ecology 22:760–772.

Desser, S. S., and G. F. Bennett. 1993. The genera *Leucocytozoon*, *Haemoproteus*, and *Hepatocystis*, p. 273–307. *In*: Parasitic Protozoa, Volume 4. J. P. Krier (ed.). Academic Press, New York.

Duellman, W. E., and L. Trueb. 1986. Biology of Amphibians. McGraw-Hill, New York.

Edwards, S. W. 1994. Biochemistry and Physiology of the Neutrophil. Cambridge University Press, New York.

Forbes, M. R., D. L. McRuer, and D. Shutler. 2006. White blood cell profiles of breeding American toads (*Bufo americanus*) relative to sex and body size. Comparative and Clinical Pathology 15:155–159.

Forson, D. D., and A. Storfer. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. Ecological Applications 16:2325–2332.

Foxon, G. E. H. 1964. Blood and respiration, p. 151–209. *In*: Physiology of the Amphibia. J. A. Moore (ed.). Academic Press, New York.

Giroux, I. 1999. Contamination de l'eau par les pesticides dans les régions de culture de maïs et de soya au Québec. Direction des écosystèmes aquatiques, Ministère de l'Environnement, Quebec.

Giroux, I. 2004. La présence de pesticides dans l'eau en milieu agricole au Québec. Ministère de l'Environnement, Direction de la suivi de l'état de l'Environnement. Envirodoq no. ENV/2004/0309, collection no. QE/141.

Giroux, I. 2010. Présence de pesticides dans l'eau au Québec—bilan dans quatre cours d'eau de zones en culture de maïs et de soya en 2005, 2006 et 2007 et dans des réseaux de distribution d'eau potable. Ministère du Développement durable, de l'Environnement et des Parcs, Direction du suivi de l'état de l'environnement.

Gross, W. B., and H. S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Diseases 27:972–979.

Hoole, D. 1997. The effect of pollutants on the immune response of fish: implications for helminth parasites. Parassitologia 39:219–225.

Houlahan, J. E., C. S. Findlay, B. R. Schmidt, A. H. Meyer, and S. L. Kuzmin. 2000. Quantitative evidence for global amphibian declines. Nature 404:752–755.

Jackson, D. A. 1993. Stopping rules in principal components analysis: a comparison of heuristical and statistical approaches. Ecology 74:2204–2214.

Kiesecker, J. M. 2002. Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? Proceedings of the National Academy of Sciences of the United States of America 99:9900–9904.

Kim, B., T. G. Smith, and S. S. Desser. 1998. The life history, host-specificity of *Hepatozoon clamatae* (Apicomplexa: Adeleorina) and ITS-1 nucleotide sequence variation of *Hepatozoon* species of frogs and mosquitoes from Ontario. Journal of Parasitology 84:789–797.

King, K. C., A. D. Gendron, J. D. McLaughlin, I. Giroux, P. Brousseau, D. Cyr, S. M. Ruby, M. Fournier, and D. J. Marcogliese. 2008. Short-term seasonal changes in parasite community structure in northern leopard froglets (*Rana pipiens*) inhabiting agricultural wetlands. Journal of Parasitology 94:13–22.

King, K. C., J. D. McLaughlin, M. Boily, and D. J. Marcogliese. 2010. Effects of agricultural landscape and pesticides on parasitism in native bullfrogs. Biological Conservation 143:302–310.

King, K. C., J. D. McLaughlin, A. D. Gendron, B. D. Pauli, I. Giroux, B. Rondeau, M. Boily, P. Juneau, and D. J. Marcogliese. 2007. Impact of agriculture on the parasite communities of northern leopard frogs (*Rana pipiens*) in southern Quebec, Canada. Parasitology 134:2063–2080.

Knutson, M. G., W. B. Richardson, D. M. Reineke, B. R. Gray, J. R. Parmelee, and S. E. Weick. 2004. Agricultural ponds support amphibian populations. Ecological Applications 14:669–684.

Lehman, C. M., and B. K. Williams. 2010. Effects of current-use pesticides on amphibians, p. 167–202. *In*: Ecotoxicology of Amphibians and Reptiles. Second edition. D. W. Sparling, G. Linder, C. A. Bishop, and S. Krest (eds.). SETAC Press, Pensacola, Florida.

Maniero, G. D., and C. Carey. 1997. Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. Journal of Comparative Physiology B 167:256–263.

Marcogliese, D. J., K. C. King, H. M. Salo, M. Fournier, P. Brousseau, P. Spear, L. Champoux, J. D. McLaughlin, and M. Boily. 2009. Combined effects of agricultural activity and parasites on biomarkers in the bullfrog, *Rana catasbeiana*. Aquatic Toxicology 91:126–134.

Norris, K., and M. A. Evans. 2000. Ecological immunology: life history trade-offs and immune defense in birds. Behavioral Ecology 11:19–26.

Rohr, J. R., and K. A. McCoy. 2010. A quantitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environmental Health Perspectives 118:20–32.

Rohr, J. R., A. M. Schoffthoefer, T. R. Raffel, H. J. Carrick, N. Halstead, J. T. Hoverman, C. M. Johnson, L. B. Johnson, C. Lieske, M. D. Piwoni, P. K. Schoff, and V. R. Beasley. 2008. Agrochemicals increase trematode infections in a declining amphibian species. Nature 455: 1235–1239.

Romanova, E. B., and M. N. Egorikhina. 2006. Changes in hematological parameters of *Rana* frogs in a transformed urban environment. Russian Journal of Ecology 37:188–192.

Romanova, E. B., and O. Y. Romanova. 2003. Peculiarities of leukocytic formula of peripheral blood of green frogs under conditions of anthropogenic load. Journal of Evolutionary Biochemistry and Physiology 39:480–484.

Rondeau, B. 2005. Water quality in the fluvial section—contamination by toxic substances, second ed. Fact sheet in the series "Monitoring the State of the St. Lawrence River". Minister of the Environment and Ministre de Développement durable, de l'Environnement et des Parcs de Québec. Available online: http://www.planstlaurent.qc.ca/sl_obs/sesl/publications/fiches_indicateurs/qualite_eau_toxique_2005_e.pdf

Rouf, M. A. 1969. Hematology of the leopard frog, *Rana pipiens*. Copeia 1969:682–687.

Shutler, D., C. D. Ankney, and D. G. Dennis. 1996. Could the blood parasite *Leucocytozoon* deter mallard range expansion? Journal of Wildlife Management 60:569–580.

Shutler, D., A. Mullie, and R. G. Clark. 2004. Tree swallow reproductive investment, stress, and parasites. Canadian Journal of Zoology 82:442–448.

Shutler, D., T. G. Smith, and S. R. Robinson. 2009. Relationships between leucocytes and *Hepatozoon* spp. in green frogs, *Rana clamitans*. Journal of Wildlife Diseases 45:67–72.

Smith, T. G. 1996. The genus *Hepatozoon* (Apicomplexa: Adeleina). Journal of Parasitology 82:565–585.

Smith, T. G., B. Kim, H. Hong, and S. S. Desser. 2000. Intraerythrocytic development of species of *Hepatozoon* infecting ranid frogs: evidence for convergence of life cycle characteristics among apicomplexans. Journal of Parasitology 86:451–458.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Tierney, K. B., A. P. Farrell, and C. J. Kennedy. 2004. The differential leucocyte landscape of four teleosts: juvenile *Oncorhynchus kisutch*, *Clupea pallasi*, *Culaea inconstans* and *Pimephales promelas*. Journal of Fish Biology 65:906–919.

Vleck, C. M., N. Vertalino, D. Vleck, and T. L. Bucher. 2000. Stress, corticosterone, and heterophil to lymphocyte ratios in free-living Adelie penguins. Condor 102: 392–400.

Voccia, I., B. Blakeley, P. Brousseau, and M. Fournier. 1999. Immunotoxicity of pesticides: a review. Toxicology and Industrial Health 15:119–132.

Vredenburg, V. T., R. A. Knapp, T. S. Tunstall, and C. J. Briggs. 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. Proceedings of the National Academy of Sciences of the United States of America 107:9689–9694.

Work, T. W., R. A. Rameyar, G. H. Balazs, C. Cray, and S. P. Chang. 2001. Immune status of free-ranging green turtles with fibropapillomatosis from Hawaii. Journal of Wildlife Diseases 37:574–581.

Wright, K. M. 2001. Amphibian hematology, p. 129–146. *In*: Amphibian Medicine and Captive Husbandry. K. M. Wright and B. R. Whitaker (eds.). Krieger, Malabar, Florida.

Note added in proof: Pesticide data for 2006 recently have been made available to us (M. Rondeau, Environment Canada, pers. comm.). Levels of atrazine and metolachlor were similar to those presented in Table 1, except for metolachlor at Île de la Commune, which was a maximum of 0.61 μg/L in late May, just after tadpoles hatched. This value is still an order of magnitude below the CCME Water Quality Guidelines for the protection of aquatic life. The range of values from June and July at that site (0.01–0.02 μg/L) was below that reported in Table 1.