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EFFECTS OF AGRICULTURAL PESTICIDES ON THE IMMUNE SYSTEM OF *RANA PIPIENS* AND ON ITS RESISTANCE TO PARASITIC INFECTION

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Abstract—In the past 30 years, many amphibian species have suffered population declines throughout the world. Mass mortality have been frequently reported, and in several instances, infectious diseases appear to be the cause of death. The role that contaminants could play in these die-offs through immunotoxic effects has been poorly investigated. In this study, juvenile leopard frogs (*Rana pipiens*) were exposed for 21 d to a mixture of six pesticides (atrazine, metribuzin, aldicarb, endosulfane, lindane, and dieldrin) and subsequently challenged with a parasitic nematode, *Rhabdias ranae*. Exposure to the mixture at environmentally realistic concentrations significantly reduced lymphocyte proliferation. Three weeks after the end of the exposure, lymphocyte proliferation had recovered and was stimulated in frogs challenged with parasites with the exception of those previously exposed to the highest concentration. No pesticide effects on phagocytosis and splenocyte numbers were detectable at the end of the exposure period, but these two parameters were diminished 21 d after the infection challenge in frogs previously exposed to the highest levels of pesticides. In these animals, the prevalence of lung infection by *R. ranae* also tended to be higher. These results suggest that agricultural pesticides can alter the immune response of frogs and affect their ability to deal with parasitic infection.

Keywords—Pesticides Amphibians Parasites *Rana pipiens* *Rhabdias ranae*

INTRODUCTION

Since the 1970s, significant declines in amphibian populations have been observed throughout the world [1]. This is a recognized phenomenon, and the patterns of losses seem to exceed normal population fluctuations [1,2]. Several causes, such as ultraviolet (UV) radiation [3], climate changes [4], pathogens [5,6], introduction of exotic species [7], habitat losses [8,9], pollution, and pesticides [10–16] have been suggested for these declines. It is likely that no single factor or group of factors has been the causative agent of these declines throughout the world; each locality may have its own particular cause or causes [12].

Observations of amphibian local die-offs have been associated with infections by different kinds of pathogens [5,17,18]. Environmental changes severe enough to be directly lethal are not necessary to cause the demise of amphibian populations; sublethal exposures could be sufficient to cause immunosuppression and an increased vulnerability to infections [19].

It is well documented today that a wide variety of stressors, including UV radiation, can cause immunosuppression in mammals [20,21]. Ultraviolet radiation has not been directly linked with immunosuppression in amphibians, but other stressors, such as high pH [19] and cold [22,23], have been shown to cause an alteration in the immune system of anurans. Limited data concerning immunotoxicity studies with pesticides are available for these animals. Most reports on that subject examined the effects of toxicants for 96 h on anuran embryos [24]. These data help us understand the immunotoxicity of different contaminants on amphibians, but they do not represent realistic exposures in the environment. Even though only a few studies evaluated the effects of contaminants on the immune system and on the resistance of amphibians against pathogens [25–28], it is clear from other animal studies that many chemicals, including pesticides, can alter their immune system either morphologically or functionally [29–31]. An immunomodulation caused by the action of contaminants could subsequently reduce the ability of these animals to defend themselves against invading pathogens [32,33].

To test the hypothesis that agricultural pesticides modulate immune responses and increase the sensitivity of amphibians to parasites, we conducted in vivo experiments on *Rana pipiens*, a frog species indigenous to North America. We exposed the frogs to different concentrations of a specific mixture of pesticides and then challenged the animals with larvae of a skin-penetrating parasite, *Rhabdias ranae*, a nematode commonly found in semiterrestrial ranid frogs [34]. Larvae of *R. ranae* penetrate the skin and migrate to the lungs, where they become hermaphroditic adults capable of producing eggs. These eggs are coughed up and swallowed before hatching in the gut and being passed in the feces. Larvae then molt and develop into free-living adult males or females. They copulate, the male dies, and the female gives birth to infective larvae [34].

The mixture of pesticides used in this experiment contained four insecticides (lindane, dieldrin, endosulfan, and aldicarb) and two herbicides (metribuzin and atrazine). Most of these pesticides were selected because of their presence in surface water at different sites along the St. Lawrence River (QC, Canada) [35,36]. The lower concentrations selected were chosen as common exposure levels in the environment, and the

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Table 1. Pesticide concentration ranges found in different tributaries of the St. Lawrence River draining intensively cultured areas in southern Quebec (Canada) during the summer

Pesticides	Concentration ranges	Rivers	1.0× Concentrations	References
Atrazine	0.10–29.00 μg/L	Chibouet River	21 μg/L	[35]
Metribuzine	0.00–0.76 μg/L	Saint-Régis River	0.56 μg/L	[35]
Endosulfane	<0.4–0.4 ng/L	Richelieu	0.02 μg/L	[36]
Lindane	<0.4–4.2 ng/L	De la Tortue River	0.33 ng/L	[36]
Aldicarb	Not detected (detection limit 100 ng/L)	Richelieu, Nicolet, de la Tortue, and Yamaska Rivers	17 μg/L	[36]
Dieldrin	<0.4 ng/L	L'Assomption, Richelieu, and de la Tortue Rivers	0.15 ng/L	[36]

higher concentrations were selected as an extreme of what could potentially occur in highly contaminated environments. These pesticides are well studied and are known to cause damage when used individually [37–42]. However, to our knowledge, their combined effect on the immune system has never been investigated.

MATERIALS AND METHODS

Animals and tissue collection

Tadpoles of *R. pipiens* at Gosner stage 25 were captured from a nonagricultural pond (free of agricultural pesticide contamination) in Boucherville (QC, Canada) and brought to the laboratory. The tadpoles were placed into tanks filled with dechlorinated tap water (temperature range 21.5–22.5°C) at a final density of approximately one tadpole per liter of water. Water was filtered with a biological filter and changed twice weekly. Tadpoles were fed ad libitum with boiled lettuce every day. The animals were kept in the tanks until metamorphosis. At metamorphosis, the juvenile leopard frogs were placed in tanks equipped with two platforms so they could get out of the water. On average, two animals were placed in each tank. They were fed live crickets (Mirdo Importations, Montreal, QC, Canada) covered with phosphorus-free carbonate calcium powder (four crickets per frog, three times a week).

Animals were sacrificed using a solution of 0.8% tricaine methanesulfonate (Boreal, St. Catherines, ON, Canada) in distilled water. Before the beginning of the exposure and after sacrifice, each frog was weighed and measured. Spleens were removed aseptically and homogenized in complete amphibian L-15 medium (Biomedia, Drummondville, QC, Canada). Cells from an aliquot of each suspension were stained with trypan blue and counted. Cell suspensions were then adjusted to the concentration required for the appropriate assay.

Chemicals

The chemicals used in the experiments were purchased from ChemService (West Chester, PA, USA). These were atrazine ($C_8H_{14}C_1N_5$), metribuzin ($C_8H_{14}N_4OS$), endosulfan ($C_{12}H_6C_{14}O_2S$), lindane ($C_6H_6C_{16}$), dieldrin ($C_{12}H_8C_{16}O$), and aldicarb ($C_7H_{14}N_2O_2S$). Atrazine stock solution was prepared in deionized water with the help of a sonicator at a concentration of 30 mg/L. Metribuzine and aldicarb stock solutions were prepared in deionized water, respectively, at concentrations of 110.01 and 3,060 mg/L. Dieldrin, endosulfane, and lindane stock solutions were prepared in 0.01% dimethyl sulfoxide (DMSO), a substance used as a vehicle for lipophilic pesticides, respectively, at concentrations of 30, 4, and 66 μg/L. The DMSO stock solution was prepared in deionized water at a concentration of 0.01%. Control and test solutions were thoroughly mixed prior to use.

Toxicity testing

Five different treatment groups were used for the experiment: control with clean water, control with DMSO, 0.1*X*, 1.0*X*, and 10*X*, where *X* represents the concentration of pesticide detected in the surface water of St. Lawrence River tributaries in which agricultural fields are drained [35,36] (atrazine: 1*X* = 21 μg/L; metribuzin: 1*X* = 0.56 μg/L; aldicarb: 1*X* = 17 μg/L, no longer found in the environment at this concentration; dieldrin: 1*X* = 0.15 ng/L; endosulfan: 1*X* = 0.02 ng/L; lindane: 1*X* = 0.33 ng/L). Comparison with pesticides levels detected in St. Lawrence River tributaries draining agricultural areas demonstrated that the 0.1*X* and 1*X* concentrations were environmentally realistic except for aldicarb (Table 1). One hundred frogs ($n = 20$ for each treatment) were exposed for 21 d. Ten frogs from each group were sacrificed 21 d after the beginning of the exposures to test different immune parameters. The remaining 50 frogs were exposed individually to 30 *R. ranae*, obtained from six naturally infected adult frogs. The larvae were placed on a moistened filter paper within a stender dish where the frogs were confined individually for 24 h. After the infection, frogs were kept for 21 d in clean water. Their feces were examined daily to determine the time required for the establishment of reproducing nematodes in their lungs (prepatent period). Following this period of 21 d, they were sacrificed to test their immune functions as well as to verify their degree of infection. A detailed protocol for infection of *R. pipiens* is available in A.D. Gendron et al. (unpublished data).

Since *R. pipiens* needs both an aquatic and a terrestrial environment, the frogs were kept for 16 h per day in aquaria containing 1 L of contaminated water and for the remaining 8 h in inclined aquaria containing a small volume of clean water and a dry platform. Each aquarium was equipped with a cover to minimize water evaporation. Test solutions were renewed three times a week by removing the total content of water in the tanks and adding an equal volume of fresh solutions. Mortality was recorded daily. Ambient temperatures ranged from 20 to 22°C. The photoperiod simulated conditions in southern Québec (Canada) during mid-June to mid-July.

Determination of cell concentration and viability

A drop of each cell suspension was introduced into an improved Neubauer hemocytometer (Scientific Products, Piscataway, NJ, USA). Total number of cells was determined microscopically. In parallel, cell viability was determined by microscopy, observing the membrane permeability of cells to trypan blue.

Immunological assays

The methods used in these experiments have been adapted from the *Manual of Immunological Methods* [43]. Cell con-

centrations, incubation time and temperature, mitogen concentrations, cell-to-bacteria ratio, and speed of centrifugation have been adjusted for the frog species. Preliminary experiments were carried out to determine the optimal conditions for each assay.

Phagocytosis

Spleen cells (1×10^5 cells/500 μl of L15) were mixed with fluorescent (fluorescein, FITC) *Escherichia coli* K-12 (Molecular Probe, Eugene, OR, USA) at a ratio of 1:25 (phagocytes:bacteria). The mixture was incubated at 29°C for 1 h, after which 25 μl of trypan blue were added to the cell suspensions in order to quench the fluorescence of bacteria that were not internalized. Negative controls were obtained by incubating the cells without bacteria. The fluorescence of engulfed bacteria was then analyzed by flow cytometry. The fluorescence emission was collected at 520 nm. A FACScan (Becton Dickinson, San Jose, CA, USA) with an air-cooled argon laser providing an excitation at 488 nm was used. A total of 10,000 events were acquired for each sample. The data were then analyzed from a dot plot showing two parameters: size (forward scatter [FSC]) on the *y* axis and complexity (side scatter [SSC]) on the *x* axis. On this dot plot, the phagocytic cell populations were isolated from the splenocytes in order to analyze their phagocytic activity. From this plot, a green fluorescence emission frequency distribution histogram was obtained, which is a direct representation of the cells' phagocytic activity. Data collection and analysis were performed with the WinMDI 2.8 software (Scripps Research Institute, La Jolla, CA, USA).

Lymphocyte proliferation assay

Spleen cells (60,000 cells/100 μl of L15 + 0.05% mercaptoethanol) were mixed with 100 μl of single mitogen in round-bottom microwell plates. Final concentration of each mitogen was 5 μg/ml for ConA (concanavilin A) and 2.5 μg/ml for PHA (phytohemaglutinin). Microplates were incubated for 72 h at 29°C with 5% CO_2. Eighteen hours before the end of the incubation, 0.5 μCi of ^{3}H-methyl thymidine (ICN, Costa Mesa, CA, USA) was added to each well. The DNA was then captured on filters and transferred to scintillation vials. The incorporated ^{3}H-methyl thymidine was measured with a β scintillation counter. The results are expressed in disintegrations per minute (DPM).

Statistical analysis

Spleen cellularity, splenocyte viability, number of active phagocytes, and lymphocyte proliferation were compared among treatment groups at the end of the exposure period and 21 d after the infection challenge using one-way analysis of variance (ANOVA). When significant difference was detected at $p < 0.05$, the means were compared using the Tukey–Kramer test. The combined effect of treatment and parasitic infection were also examined by means of a two-way ANOVA with interaction. A Fisher's exact test was finally performed to evaluate the differences in prevalence of *Rhabdias* larvae among groups, defined as being the percentage of frogs infected by one or more *R. ranae* in a given group. Data were analyzed using the SAS system for Windows Version 8 (SAS Institute, Cary, NC, USA). The assumption of normality and homoscedasticity were verified before each ANOVA.

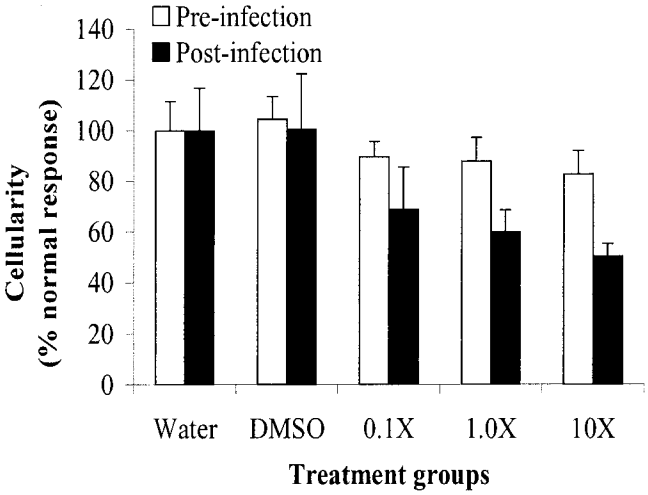

Fig. 1. Effect of an exposure to pesticides and subsequently to parasites on the cellularity of frog splenocytes. Cellularity results are expressed in the percentage of normal response ([number of cells in spleen of exposed frogs/number of cells in spleen of frogs exposed to clean water] × 100). Bar = mean; error bar = standard error of means; DMSO = dimethyl sulfoxide.

RESULTS

Effects of exposure to pesticides and subsequently to parasites

Viability of frog splenocytes. Exposure of frogs to pesticides and subsequently to parasites did not significantly alter the viability of splenocytes. Viability of splenocytes before the infection was around 80%, while viability after the infection was around 90%.

Cellularity of frog splenocytes. Exposure of frogs to pesticides and subsequently to parasites did not significantly alter the number of splenocytes ($p = 0.433$, $F = 0.95$, $df = 4$; Fig. 1). Although a tendency toward a lower cellularity is observed as the concentration of pesticides increases, no significant difference was measured among treatment groups. However, infected frogs exposed to the 10*X* concentration possessed a cellularity that tended to be lower ($29.3 \times 10^4 \pm 2.91$ cells/ml) than infected frogs exposed to clean water ($58.15 \times 10^4 \pm 9.82$ cell/ml) and DMSO ($58.5 \times 10^4 \pm 11.14$ cells/ml) ($p = 0.069$, $F = 2.35$, $df = 4$).

Lymphocyte proliferation of frog splenocytes. Exposure of frogs to pesticides significantly reduced the proliferation of T-lymphocytes in response to mitogens (ConA and PHA) at all concentrations tested compared to the proliferation observed in frogs exposed to DMSO (ConA: $p = 0.0009$, $F = 6.621$, $df = 4$; PHA: $p = 0.0018$, $F = 5.870$, $df = 4$; Fig. 2A). Moreover, the proliferation of splenocytes in the absence of mitogens was significantly reduced in all frogs exposed to pesticides compared to frogs exposed to DMSO ($p = 0.0001$, $F = 12.42$, $df = 4$). However, in comparison to animals exposed to clean water, the proliferation of splenocytes in the absence of mitogen was reduced only in frogs exposed to the highest concentration of pesticides (10*X*), and the proliferation of T-lymphocytes in response to mitogens was not reduced in animals exposed to pesticides. Effects of pesticides on B-lymphocyte proliferation remain to be determined since no adequate mitogens were found to stimulate the proliferation of frog B-lymphocytes.

Compared to noninfected frogs, all frogs infected with *R. ranae* after pesticide exposure had higher T-lymphocyte pro-

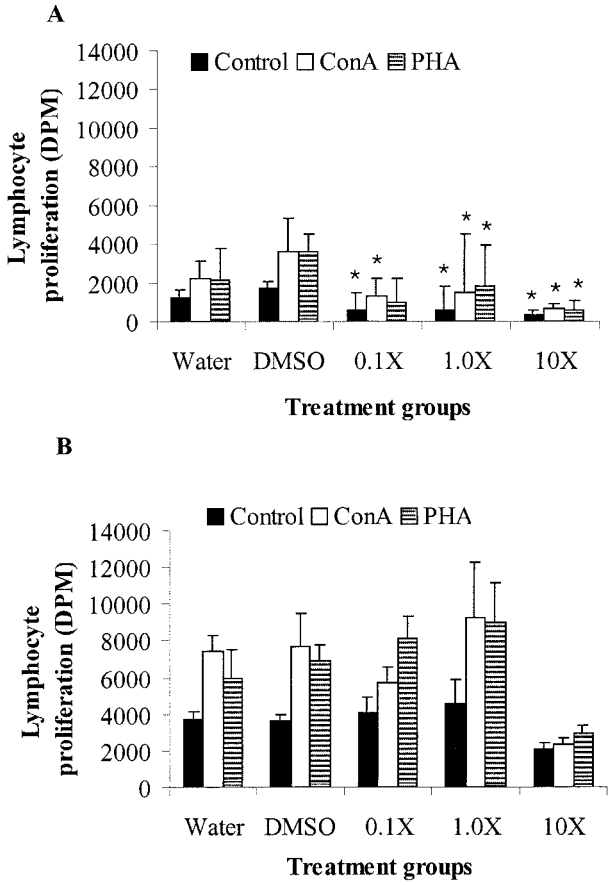

Fig. 2. Effect of an exposure to pesticides and subsequently to parasites on the proliferation of T-lymphocytes present in frog spleens. Results are expressed in disintegration per minute (DPM). Bar: mean; error bar: standard error of means. For each panel, significant difference compared to the corresponding dimethyl sulfoxide (DMSO) group is represented by an asterisk. (**A**) Proliferation of lymphocyte in frogs following the exposure to pesticides alone. (**B**) Proliferation of lymphocytes following the exposure to pesticides and subsequently to parasites. A two-way analysis of variance was performed to test the combined effect of pesticides and parasite infection on the proliferation of lymphocytes with and without stimulation by mitogens. The *p* values associated with the effects retained by the model are as follow: control: treatment ($p = 0.036$), parasite ($p < 0.001$); ConA (concanavilin A): treatment ($p = 0.009$), parasite ($p < 0.001$); phytohemaglutinin (PHA): treatment ($p = 0.013$), parasite ($p < 0.001$).

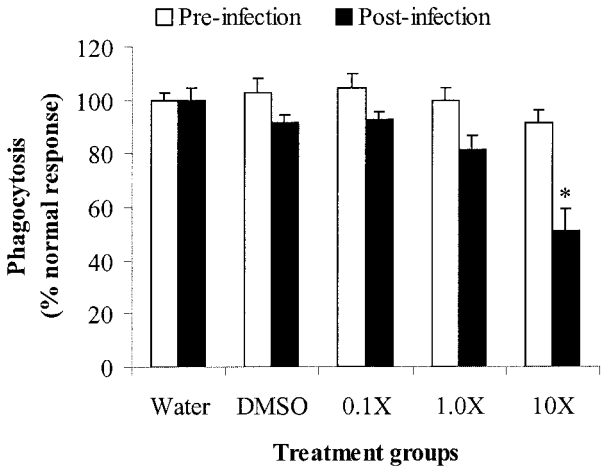

Fig. 3. Effect of an exposure to pesticides and subsequently to parasites on the number of frog phagocytic splenocytes. Results are expressed in the percentage of normal response ([number of phagocytes present in the spleens of exposed frogs/number of phagocytes present in the spleen of frogs exposed to clean water] × 100). Bar: mean; error bar: standard error of means. The asterisk indicates significant difference compared to the corresponding dimethyl sulfoxide (DMSO) group as found by the one-way analysis of variance (ANOVA) followed by a Tukey–Kramer test. Results of a two-way ANOVA performed to test the combined effects of pesticide treatment and parasite infection are also shown.

liferation (compare values in Fig. 2A and B). Moreover, infected frogs exposed to a high concentration of pesticides (10*X*) tended to have a nonsignificant reduction of T-lymphocyte proliferation (in response to ConA) compared to the other groups of infected frogs ($p = 0.068$, $F = 2.50$, $df = 4$). However, no significant difference was measured in the proliferation of T-lymphocytes (without mitogen and in response to PHA) from other infected frogs (without mitogens: $p = 0.187$, $F = 1.676$, $df = 4$; PHA: $p = 0.192$, $F = 1.672$, $df = 4$). A two-way ANOVA indicated that pesticides as well as parasites had an independent effect on the immune system.

Number of frog phagocytic splenocytes. Exposure of frogs to pesticides did not significantly alter the percentage of active phagocytes in the spleens of frogs (Fig. 3). Although a decrease in the percentage of phagocytes is observed as the concentration of pesticides increases, no significant difference was measured between the different treatment groups ($p = 0.352$, $F = 1.14$, $df = 4$). However, following the infection of frogs with *R. ranae*, the percentage of active phagocytes was found to decrease significantly in frogs exposed to the 10*X* concentration of pesticides (1727.89 ± 179.26 cells) compared to water (3897.5 ± 128.27 cells) and DMSO (3662.61 ± 185.7 cells) control frogs ($p < 0.001$, $F = 13.06$, $df = 4$; Fig. 3). A two-way ANOVA, computed to test the combined effects of pesticide exposure and parasite infection, found an interaction between pesticide treatment and parasite exposure (pesticide treatment × parasite). The effect of parasite infection on phagocytosis varies according to the concentration of pesticide used in the assay. Two-way ANOVA results are presented in Table 2.

Effects of pesticides on the infection parameters of R. ranae

The prevalence of lung infection by *R. ranae* tended to be higher in all treated groups compared to DMSO. Prevalence of infection was 70% in DMSO control (80% in clean water), whereas in frogs exposed to 0.1*X*, 1.0*X*, and 10*X* concentrations of pesticides, prevalence was 80, 100, and 100%. However, among-group differences were not found significant by a Fisher's exact test ($p = 0.2304$).

DISCUSSION

In this study, we examined the effects of a representative mixture of pesticides on the immune system of frogs before and after exposure to a parasitic nematode infection. Even though the mixture of pesticides did not have any effect on the cellularity and phagocytic activity of cells present in the spleen, our results suggest that a short-term administration of pesticides can have an effect on other immune functions of anurans. Indeed, when frogs were exposed to 0.1*X*, 1.0*X*, and 10*X* concentration of pesticides, T-cell proliferation was found to be significantly reduced compared to the DMSO control animals. T-cells have a central role in the defense against extracellular parasites. Indeed, in vitro T-lymphocyte proliferation responses seem to correlate with immunity to certain nem-

Table 2. Two-way analysis of variance (ANOVA) summary of measures obtained following the exposure of *Rana pipiens* to either pesticides alone or to parasites and pesticides

	Pesticides			Parasites			Pesticide × parasite[a] interaction		
	F[b]	*p*[c]	*df*[d]	*F*	*p*	*df*	*F*	*p*	*df*
Phagocytosis	11.04	<0.0001	4	26.82	<0.0001	4	4.29	0.0032	4
Lymphocyte proliferation									
Without mitogen	2.79	0.0357	4	58.79	<0.0001	4	—	—	4
Concanavilin A	3.74	0.0092	4	35.00	<0.0001	4	—	—	4
Phytohemaglutinin	3.52	0.0131	4	45.89	<0.0001	4	—	—	4

[a] See text for explanation.
[b] Results of ANOVA *F* test.
[c] Significance probability.
[d] Degrees of freedom.

atodes, such as *Haemonchus contortus* in sheep [44], and T-lymphocytes are responsible for the activation of B-cells, which secrete molecules involved in parasitic defense [44,45]. Once activated, specific frog B-cells can differentiate into plasma cells capable of secreting highly specific antibodies, such as IgX and IgY, which seem to be, respectively, the equivalent of IgA and IgG in mammals [11]. These antibodies, especially IgXs, could be important in mucosal defense when the parasites are present in the throat or the intestine. It has already been observed in mammals that other nematodes increase significantly the number of IgA-producing cells, suggesting an important role for this immunoglobulin in resistance to nematode infection [44]. An inhibition of T-cell proliferation will therefore impair the activation of B-cells, and secretion of these antibodies (IgX and IgY) will also be reduced, which means that the nematodes could have a greater facility to invade the body.

The reduction of certain immunological responses in the exposed groups might explain in part the increase of prevalence of *R. ranae* in these groups. Actually, prevalence of the parasite in the lungs increased with contaminant doses up to 100% at 1.0*X* and 10*X* pesticide concentrations, suggesting that these frogs have no resistance to infection, whereas in a number of control frogs (treated with DMSO or clean water), not all nematodes established in the lungs by the end of the experiment. Moreover, frogs exposed to the 10*X* pesticide concentration had a significantly greater number of nematodes in their lungs 21 d after the infection challenge compared to water and DMSO control animals (results presented in A.D. Gendron et al., unpublished data).

Boroskova et al. [46] obtained similar results in an experiment conducted on guinea pigs. Animals were exposed to heavy metal emissions and further challenged with the parasite *Ascaris suum*. The authors observed an increase in the number of migrating larvae in animals following an exposure to contaminants. They suggested that exposure to toxicants caused alterations of immune mechanisms important during the migration phase of *A. suum* larvae. During this phase, antigen recognition is necessary for the immune system to combat the parasites. An inhibition of antigen recognition or presentation might therefore be the explanation for the reduced lymphocyte proliferation observed in the frogs exposed to the 10*X* pesticide concentration.

It is interesting to note that the immunological responses observed were not the same prior and after infection with the larvae. Indeed, spleen cellularity, lymphocyte proliferation, and the number of active phagocytes were not the same before and after infection. The spleen cellularity decreased after infection with *R. ranae* in frogs that were exposed to pesticides. Normally, after an infection, the immune system is primed, and the cells of this system are proliferating and dividing in order to fight the invading pathogens. Following an infection, an increased number of cells in the spleen would therefore be expected. However, as the concentration of pesticides to which frogs were exposed prior to infection increased, the number of cells present in the spleen tended to decrease. A two-way ANOVA indicated that both the pesticides and the parasites alone had an effect on the cellularity. However, no interaction between these two factors was apparent. This might indicate that the mixture used creates delayed long-term damage on the immune system that can be seen only a few weeks after exposure. Moreover, the parasite treatment on its own decreased the number of cells in the spleen for an unknown reason.

An overall increase was observed in the stimulated (with mitogens) and unstimulated levels of lymphocyte proliferation in frogs from all treatment groups 21 d after the infection challenge. Contrary to what was observed in frogs exposed to pesticides only, T-cell responses in the 0.1*X* and the 1.0*X* pesticide treatments were comparable to those observed in the DMSO and the clean water–infected groups, demonstrating that lymphocytes from exposed and subsequently infected frogs were able to respond normally to mitogens. Conversely, T-cell proliferation was only weakly stimulated by mitogens in infected frogs previously exposed to the 10*X* pesticide concentration. This indicates that 21 d after an infection challenge, infected animals previously exposed to a high concentration of pesticides remained unable to respond adequately to mitogens. Twenty-one days after exposure was terminated, the frogs' immune system would have recuperated and matured, which likely explained the improved response of T-lymphocytes to mitogen challenge and the increase in lymphocyte proliferation observed in water-, DMSO-, 0.1*X*-, and 1.0*X*-infected groups. This would imply that pesticides have a short-term effect in low concentration exposures. The weak response observed in infected animals exposed to the 10*X* pesticide concentration might therefore indicate that their immune system has suffered long-term damage from exposure to pesticides. In order to determine if the mixture used can have permanent effects on this system, longer periods of recuperation after toxic exposure would be required. Alternatively, lymphocyte proliferation in the water-, DMSO-, 0.1*X*-, and 1.0*X*-infected groups could have increased in response to infection. This would then imply that infected animals previously ex-

posed to the highest concentration of pesticides (10*X*) were not able to respond adequately against parasites. The two experiments mentioned in this paper (exposure to pesticides and then to parasites) should be done at the same time to understand better what are the effects of each treatment on the immune system of anurans.

No significant difference was observed among groups in the percentage of active phagocytes before the infection. However, 21 d after the infection, the frogs exposed to the 10*X* pesticide concentration showed a decrease in the percentage of active phagocytes present in their spleens. The explanation for these results is still not clear. A two-way ANOVA indicates that the parasites and the pesticides have an interactive effect on the immune system. Similar effects have also been observed by Boroskova et al. [47]. It was observed that exposure of guinea pigs to either heavy metals or *A. suum* alone did not have an effect on some immune functions, such as the classical complement pathway. However, when the animals were exposed to heavy metals and further to parasites, a decrease in this function was observed. Therefore, these results suggest that pollutants and parasites were interacting together to alter immune function. It is likely that other parasites, such as *R. ranae*, could have a similar effect on the immune system when combined with pesticides. Parasites can alter the immune response in various ways to escape the immune surveillance [48]. The strategy of the parasite is often to develop a niche in the host in which they are able to survive and reproduce without significantly disrupting the viability of the organism that they have infected [48]. Therefore, their effects alone often do not suppress the immune system enough to impair the normal responses of the host. However, it is possible that when their suppressive effects are combined with the ones induced by pesticides, the immune responses of the host might be exhausted. Exposure to pollutants followed by an infection might complicate the immune system reactions, thus further inhibiting an adequate defense against other pathogens.

Finally, DMSO alone seems to have an effect on the immune cells. Indeed, DMSO increased the proliferation of T-lymphocytes in *R. pipiens* following a 21-d exposure to pesticides. The reason why this substance modulates this immune function is not known. Further studies would be needed to understand its action on the immune system. Even though DMSO has some effects on frogs, these effects are not as important as the one created by other carriers, such as methanol. In previous experiments, methanol was used to dissolve certain pesticides in our mixture. This carrier caused sex reversal in developing frogs and had a negative effect on the survival of animals (unpublished data). Therefore, although DMSO affects the immune system of frogs, it remains an acceptable carrier. It does not seem to affect the development and growth of anurans, and we can determine its effect on the immune system of frogs by comparing the competence of animals exposed to clean water with the ones of animals exposed to DMSO.

Our study will help provide a general understanding on the impact of agricultural practices on aquatic systems. Our results suggest that pesticides can increase the susceptibility of organisms to pathogens by depressing their immune system. Furthermore, parasites and pathogens may become more virulent in immunocompromised individuals (Gendron et al., in preparation). Given that animals exposed to pollutants have increased energy consumption associated with detoxification and elimination, less energy is available to combat different pathogens [49]. Moreover, pathogens and pesticides might have a synergistic effect on the immune system. This is of particular concern since most frogs living in the wild are infected with various parasites and pathogens that are normally not lethal. If pollutants increase immunosuppression in infected frogs, it may render the animals more vulnerable to a variety of infections. It is then clear that the interaction between pesticides, parasites, and the immune system deserves further study. This may contribute to further understanding the causes responsible for the decline in amphibian populations observed since the 1970s.

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