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## Exposure of leopard frogs to a pesticide mixture affects life history characteristics of the lungworm *Rhabdias ranae*

Received: 26 July 2002 / Accepted: 31 January 2003 / Published online: 18 March 2003  
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**Abstract** We tested the hypothesis that exposure of leopard frogs (*Rana pipiens*) to agricultural pesticides can affect the infection dynamics of a common parasite of ranid frogs, the lungworm *Rhabdias ranae*. After a 21-day exposure to sublethal concentrations of a pesticide mixture composed of atrazine, metribuzin, aldicarb, endosulfan, lindane and dieldrin, or to control solutions (water, dimethyl sulfoxide), parasite-free juvenile frogs were challenged with 30 infective larvae of *R. ranae*. Approximately 75% of the larvae penetrated the skin and survived in both exposed and control animals, suggesting that pesticides did not influence host recognition or penetration components of the transmission process. Rather, we found that the migration of *R. ranae* was significantly accelerated in hosts exposed to the highest concentrations of pesticides, leading to the establishment of twice as many adult worms in the lungs of frogs 21 days post-infection. Pesticide treatment did not influence the growth of lungworms but our results indicate that they matured and reproduced earlier in pesticide-exposed frogs compared to control animals. Such alterations in life history characteristics that enhance parasite transmission may lead to an increase in virulence. Supporting evidence shows that certain components of the frog immune response were significantly suppressed after exposure to the pesticide mixture. This suggests that the immune system of anurans exerts a control over lungworm

migration and maturation and that agricultural contaminants can interfere with these control mechanisms. Our results also contribute to the ongoing debate regarding the role that anthropogenic factors could play in the perplexing disease-related die-offs of amphibians observed in several parts of the world.

**Keywords** Amphibian · Contaminant · Parasite infection · *Rana pipiens* · Virulence

### Introduction

Numerous amphibian species have suffered dramatic declines in many regions of the world over the last decades (Alford and Richards 1999; Houlihan et al. 2000). A number of these disappearances are clearly attributable to habitat fragmentation and destruction, but in many other cases the exact causes of decline remain obscure (Alford and Richards 1999). Most intriguing is the possible implication of infectious disease in the waves of mass mortality that decimated several anuran populations in western North America in the 1970s and in Eastern Australia, Central America and Europe in the 1990s (Laurance et al. 1996; Carey et al. 1999). So far, mass mortalities have been attributed to fungus-like pathogens, viruses, and bacteria (Cunningham et al. 1996; Carey et al. 1999; Daszak et al. 1999; Bollinger et al. 1999). It is well known that pathogen-driven mortalities contribute to the control of host populations (Esch and Fernández 1993) but the geographical spread and amplitude of the reported die-offs appear to exceed such a regulatory influence. Some authors are convinced that these mass deaths result from the exposure to emerging virulent pathogens or to existing forms recently introduced into habitats where they were not previously found and against which amphibian populations have not evolved an effective immune defense (Daszak et al. 1999). Long-term exposure to multiple anthropogenic stressors, such as pollutants, could also have weakened amphibians rendering them more vulnerable to opportu-

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nistic pathogens (Carey et al. 1999; Taylor et al. 1999). Several pollutants including pesticides have been shown to cause immunotoxicity in various animal taxa (Hoole 1997; Luebke et al. 1997). These toxicants can modulate the innate or the adaptive components of the immune system through a direct effect on immune cells (Vermeer and Hurks 1996) or indirectly via endocrine-disruptive effects (Gendron et al. 1997; Weyts et al. 1999). Currently, existing data on the immunotoxicity of xenobiotics in amphibians are scarce and only a few studies have examined their impact on the resistance of anurans to pathogens or parasites (e.g. Taylor et al. 1999; Kiesecker 2002).

This laboratory study was conducted to determine if exposure of leopard frogs to a mixture of pesticides at sublethal concentrations can alter the relationship between frog hosts and a skin-penetrating parasite, *Rhabdias ranae*. The northern leopard frog (*Rana pipiens*) is an anuran amphibian widespread in North America. Along with other ranids, this frog has experienced major declines in the 1970s, mostly in the western part of its range (Wagner 1997; Wayne and Cooper 2001) where outbreaks of diseases were reported (Carey et al. 1999). Leopard frogs are known to colonize a variety of habitats including the flood plains of rivers where they lay their eggs in early spring (Gilbert et al. 1994). In areas where agricultural activity is intense, leopard frogs are thus directly exposed to pesticides during much of their life cycle, either to waterborne chemicals carried along from drainage basins or to pesticides sprayed on fields when semi-terrestrial adults and juveniles disperse during the summer. Given that metamorphosed amphibians and older larvae are those principally dying of infectious disease in the wild (Carey et al. 1999), exposure and infection experiments were carried out on juvenile frogs. The parasite tested, *R. ranae*, is a common lungworm of North American ranids (Baker 1979a). Frogs become infected as they emerge from the aquatic habitat and come into contact with infective larvae of the parasite living in the soil. These free-living larvae penetrate the skin of frogs and migrate to the lungs where they mature (Baker 1979b). Adult frogs and toads can tolerate moderate levels of lungworm infection without much obvious impact on their health. However, when they accumulate in the lungs of juveniles, these nematodes have been shown to affect host growth and physical performance (Goater and Ward 1992; Goater et al. 1993).

## Materials and methods

### Frog rearing and maintenance

We collected recently hatched tadpoles of *R. pipiens* at a protected pristine site (Boucherville, Québec, 45°39.139'N; 73°26.678' W) and reared them in the laboratory to ensure parasite-free specimens. Tadpoles at stage 25 (Gosner 1960) were raised in glass aquaria at a water temperature of 22°C (±1°C). Biological filtration and partial water changes twice a week maintained ammonia and nitrogenous waste at safe non-toxic levels. Animals were progressively thinned

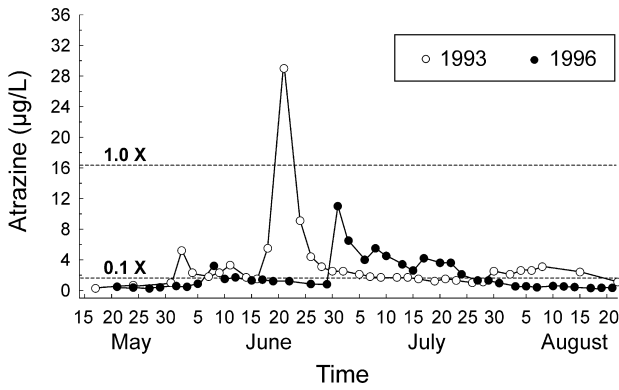
to a final density of 1 tadpole/l to attenuate the negative impact of growth inhibitors emitted by fast-growing individuals. Their diet consisted of boiled lettuce to which spinach and/or a formulated dried food (Algae wafers; Hikari, Kyorin) was periodically added as a supplemental source of protein and vitamins. Tadpoles were fed ad libitum twice daily until initiation of metamorphosis (Gosner stage 43), when they switch to a carnivorous diet. Froglets were housed in pairs in plastic containers designed with a dual level base that provided access to both wet and dry areas. After a brief non-feeding period, individuals were given live crickets of increasing size (Mirido Importations, Montreal). Each frog received four crickets 3 times a week. Before feeding, insects were dusted with a phosphorous-free calcium carbonate powder (Rep-Cal; Rep-Cal research labs, Los Gatos, Calif.) in order to avoid nutritional deficiencies caused by the low calcium:phosphorous ratio of the exoskeleton of crickets (Institute of Laboratory Animal Resources 1974). The water source for both tadpoles and juveniles was municipal drinking water subjected to purification treatments, including dechlorination and UV filtration. We regularly monitored physicochemical variables (chlorine, pH, conductivity, ammonia) to ensure fulfillment of water quality criteria. Overall, these maintenance conditions resulted in a survival rate of 92% up to Gosner stage 45. All surviving tadpoles successfully completed metamorphosis. Parasitological examination of a subsample of five froglets confirmed the absence of parasitic helminths in our experimental animals.

Adult frogs (snout-vent length 65.9–86.3 mm) collected in wetlands along the St. Lawrence River basin were also kept in the laboratory as a source of infective larvae of *R. ranae* lungworm. Animals were housed individually in plastic containers larger than those used for juveniles but similarly designed. They were fed with crickets 3 times a week and their water was renewed daily.

### Exposure to pesticides

The experimental design included the following five treatment groups: three dilutions of a representative mixture of pesticides (0.1×, 1× and 10×), a negative control (dechlorinated water) and a solvent control. The mixture was composed of six chemicals which included triazine pesticides currently in use, as well as banned organochlorine and carbamate insecticides that still persist in the aquatic environment at trace levels (Rondeau 1996; Giroux 1999). Pesticide concentrations in the 1× mixture were the following: atrazine (21 µg/l), metribuzine (0.56 µg/l), aldicarb (17 µg/l), dieldrin (0.15 ng/l), endosulfan (0.02 ng/l) and lindane (0.33 ng/l). A major constituent of the mixture, atrazine, is a triazine herbicide widely used in corn crops since the mid-1950s. Comparison with levels of atrazine detected in tributaries of the St. Lawrence River (Quebec) which drain corn crop areas indicated that the 0.1× and the 1× concentrations are environmentally realistic (Fig. 1). Solvent control animals were exposed to the same concentration of dimethyl sulfoxide (DMSO) as is found in the mixture of pesticides (5×10<sup>-6</sup>%). This chemical was used as a vehicle to dissolve lipophilic pesticides.

Prior to the onset of experiments, snout-vent length of all experimental animals was measured to the nearest 0.1 mm with an electronic caliper and their weight was recorded to the nearest 0.01 g. Four-week-old juveniles were assigned to the treatment groups to obtain five groups of 20 frogs with similar mean weight and size (Table 1). Frogs of a given treatment were housed by group of ten in two separate tanks. For the duration of the experiment, animals were placed for 16 h/day in 37.8-l glass aquaria containing enough treatment solution to immerse half their body (1 l). During the remaining 8 h/day, they were maintained in tilted tanks where they had access to either clean water or a dry platform. This exposure regimen was adopted to mimic the semi-terrestrial habitat of *Rana pipiens*. This system also facilitated feeding with crickets, which drown rapidly when no dry surface is available. Each aquarium had a lid to minimize water evaporation and prevent frogs from escaping. Test solutions were renewed 3 times a week replacing the contents of the tanks with an equal



**Fig. 1** Comparison between the concentrations of atrazine in the experimental mixture and the levels of this herbicide in surface water of rivers draining intensively cultivated areas in southern Quebec. Atrazine levels shown are those measured in 1993 (○) and 1996 (●) in the Chibouet River (data obtained from Giroux 1999, with permission from the author). Concentrations of atrazine in the 0.1x and 1x dilutions of the mixture indicated by ... Atrazine level in the 1x mixture corresponds to peak values periodically reached in the aquatic environment after intense rainfall, whereas the level in the 0.1x mixture is comparable to background levels typically detected in surface water during summer

volume of fresh solution. At the end of the 21-day exposure period, immunoassays were performed on a subsample of ten frogs of each treatment group (reported in Christin et al., in press). The remaining 10 frogs/treatment were subjected to an infection challenge.

**Infection challenge with *Rhabdias ranae***

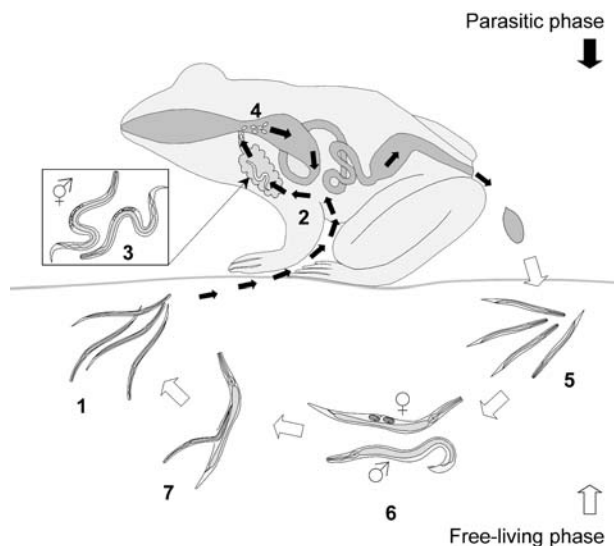
To examine the impact of pesticides on host-parasite interactions, we exposed frogs to a skin-penetrating nematode, *R. ranae*. This parasite, commonly found in the lungs of semi-terrestrial ranids, has a direct life cycle involving only one host (Baker 1979a; 1979b; Fig. 2). Larvae produced by this lungworm are expelled in frog feces, where they molt into male and female adults that mate and produce infective offspring within 5 days. We used feces from six naturally infected adult frogs as a source of infective larvae. The larvae were cultured following a modified version of Goater and Ward's (1992) procedure. Briefly, infected feces were incubated in petri dishes at room temperature (21°C) for 3 days, at which time gravid females were transferred to fresh non-infected frog feces and allowed to sit for 2 more days. Infective larvae released at incubation day 5 from degenerating females were used for the infection challenge. Feces from naturally infected frogs can be contaminated by other parasitic nematode larvae, notably *Oswaldocruzia* spp. (Trichostrongylidae), which are very difficult to distinguish from *R. ranae* larvae. The selection of gravid females of *R. ranae* at day 3 was done to minimize the risk of dual infection.

After 21 days of exposure to pesticides or control solutions, ten frogs from each treatment group were individually challenged with 30 infective larvae of *R. ranae*. The larvae were placed on a moistened filter paper within a stender dish where each frog was confined for 24 h. In a preliminary experiment, juvenile leopard frogs were exposed to various numbers of infective larvae ranging from ten to 100, and the nematodes established in the lungs after 3 weeks were enumerated for each dose. Because it yielded the most consistent results, the dose of 30 larvae was selected for the definitive study. After the infection challenge, frogs were housed individually in plastic containers with fresh water and fed as described previously. Their feces were examined daily for signs of infection with nematode larvae to determine the time required for the establishment of reproducing nematodes in their lungs (prepatent period). Frog cages were cleaned every other day to

**Table 1** Size and weight of leopard frogs assigned to five treatment groups at the onset of the experiment ( $t_0$ ), at the end of the 21-day pesticide exposure ( $t_1$ ), and 21 days after the infection challenge with *Rhabdias ranae* ( $t_2$ ). Values are presented as means (sample size, SE). Difference among treatment groups were examined using one-way ANOVA. Significance probability ( $P$ ) and  $F$  statistics are presented for  $t_0$ ,  $t_1$  and  $t_2$

Treatment groups <sup>a</sup>	Snout-vent-length (mm)			Weight (g)		
	$t_0$	$t_1$	$t_2$	$t_0$	$t_1$	$t_2$
H <sub>2</sub> O	37.78 (20, 0.69)	39.14 (20, 0.79)	41.71 (10, 0.71)	4.03 (20, 0.23)	5.45 (20, 0.36)	5.83 (10, 0.27)
DMSO	37.88 (20, 0.69)	39.29 (20, 1.00)	41.57 (10, 0.77)	4.15(20, 0.25)	5.61 (20,0.41)	6.02 (10, 0.27)
0.1x	37.63 (20, 0.52)	39.26 (20, 0.63)	41.48 (10, 0.75)	3.99 (20, 0.19)	5.33 (20, 0.25)	5.89 (10, 0.26)
1.0x	37.32 (20, 0.68)	39.43 (20, 0.79)	43.25 (10, 0.85)	4.07 (20, 0.29)	5.46 (20, 0.35)	6.53 (10, 0.30)
10x	38.04 (20, 0.65)	39.08 (20, 0.90)	42.38 (10, 0.54)	4.23 (20, 0.20)	5.27 (20, 0.33)	6.31 (10, 0.24)
One-way ANOVA	$P=0.9499, F_{4,95}=0.18$	$P=0.9986, F_{4,95}=0.03$	$P=0.4009, F_{4,45}=1.03$	$P=0.9594, F_{4,95}=0.16$	$P=0.9615, F_{4,95}=0.15$	$P=0.3289, F_{4,45}=1.19$

<sup>a</sup>Frogs were exposed to control solutions (H<sub>2</sub>O, DMSO) and to three dilutions of a pesticide mixture (x). Concentration of pesticides in the 1.0x treatment was 10 times lower than in 10x treatment and 10 times higher than in 0.1x treatment. DMSO dimethyl sulfoxide



**Fig. 2** Life cycle of *Rhabdias ranae* (Rhabditidae). The cycle includes a parasitic phase inside the frog and a free-living phase in the soil from which infective larvae arise. When in contact with a frog, infective larvae penetrate the skin (1) and migrate to the lungs (2) where they establish and become hermaphroditic adults (3). They then produce eggs which pass up the trachea, enter the gut (4) and hatch into larvae in the large intestine before they are released in feces (5). In the soil, these larvae molt into male and female adults that then mate (6). Ovoviviparous larvae that escape from degenerating females are infective (7)

prevent re-infection of individuals with parasite larvae from their own feces.

#### Tissue sampling and parasitological examination

At day 21 post-infection (p.i.), all frogs were euthanized in a solution of 0.8% tricaine methanesulfonate (MS222; Boreal, St. Catharines, Ontario). Animals were measured, weighed and sexed by examination of gonads. Their tissues were examined for the presence of parasitic helminths. Adult and subadult *R. ranae* found in the frogs were enumerated and preserved. Parasites were relaxed in hot 70% ethanol and stored in a solution of 70% ethanol containing 5% glycerol. Adult lungworms were later cleared by the progressive evaporation of ethanol in the storage solution and transferred to pure glycerol before being permanently mounted on slides in glycerol jelly. Length of worms was measured under a dissecting microscope using image analysis (Image-Pro release 4.1; Media Cybernetics, Md.).

#### Chemicals

The pesticides used in the experiments were purchased from ChemService (West Chester, Pa.). Stock solutions were prepared as follows. Atrazine was dissolved in deionized water, with the help of a sonicator at a concentration of 30 mg/l. Metribuzine and aldicarb solutions were prepared in deionized water at concentrations of 110.01 mg/l and 3060 mg/l, respectively. Dieldrin, endosulfan and lindane were mixed in 0.01% DMSO (Sigma, St. Louis, Mo.) at concentrations of 30 µg/l, 4 µg/l and 66 µg/l, respectively. Fixative and storage solutions containing alcohol were prepared using non-denatured absolute ethanol (98%) purchased from Alcools de Commerce (Varenes, QC). Waste pesticide solutions and other hazardous material were stored safely until being collected by an authorized waste management company for appropriate disposal.

#### Calculations and statistical analyses

Levels of infection by *R. ranae* are reported here as prevalence and mean abundance. Prevalence is defined as the percentage of frogs infected by one or more *R. ranae* in a given group and mean abundance as the mean number of parasitic nematodes found in infected and noninfected frogs (Bush et al. 1997). Because of the small sample size, a Fisher's exact test was used instead of a  $\chi^2$ -test to compare the prevalences of lung infection among groups. The effect of treatment on mean abundance of *R. ranae* and the time to detection of larvae in frog feces was examined using an ANOVA on untransformed data when the assumptions of normality and homoscedasticity were verified. When a significant effect was detected, the ANOVA was followed by a Ryan-Einot-Gabriel-Welsch multiple-range test (equal cell sizes). Analyses of contrast were also carried out to test hypotheses that could not be examined through multiple comparisons. An analysis of covariance (ANCOVA) tested the potential combined influence of treatment and host characteristics (sex, size and weight) on infection. The effect of treatment on worm size at the end of the experiments was also examined using an ANCOVA, with the number of *R. ranae* in the lungs of each frog as a covariate. This analysis accounted for the influence of intraspecific competition on lungworm size. Snout-vent length (svl), weight (*w*) and condition of frogs were compared among treatments using ANOVAs. The Fulton condition factor (*k*) was calculated for each individual frog as  $k=(w/svl^3)\times 10^6$ . Because frogs were exposed to treatment solutions by groups of ten instead of individually, the potential tank effect was tested for all variables measured, but always found not significant. For all tests, an  $\alpha$ -level of 0.05 was used to judge statistical significance. The homogeneity of variance and the normality of distribution were verified by means of a  $F_{max}$  test and the Shapiro-Wilk *W* statistic, respectively. Data were analyzed using SAS system release 8 for Windows (SAS Institute, Cary, N.C.).

## Results

### Influence of pesticides and parasite infection on the growth and condition of frogs

Mean snout-vent length and weight of frogs assigned to the five treatments did not differ significantly among groups at the onset of the experiment (Table 1). Although the frogs' weight and size increased during the course of the study, no difference in growth was detected among treatment groups either at the end of the exposure period or 21 days p.i. (Table 1). Similarly, differences in condition factor (data not shown) among treatment groups were not significant at any time (ANOVA,  $F_{4,95}=0.55$ ,  $P=0.6984$  at the end of the 21-day exposure;  $F_{4,45}=0.41$ ,  $P=0.8007$ , 21 days p.i.).

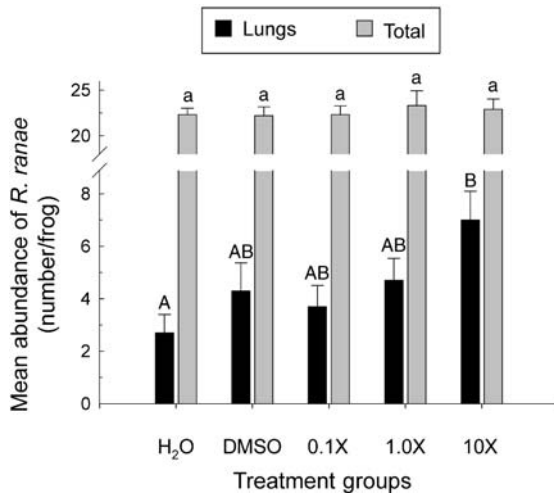
### Effect of pesticides on infection by *Rhabdias ranae*

Treatment significantly influenced the time to detection of *R. ranae* larvae in the frog feces (Table 2; ANOVA,  $F_{4,45}=3.45$ ,  $P=0.0153$ ). According to a post hoc analysis of contrast, nematodes established in the lungs and released eggs more rapidly in groups exposed to the highest concentrations of pesticides (1× and 10×) as compared to frogs exposed to control solutions (water and DMSO) ( $F_{1,45}=6.12$ ,  $P=0.0172$ ). Twenty-one days after the challenge, all frogs from the 1× and 10× groups had at

**Table 2** Dynamics of establishment and maturation of *Rhabdias ranae* in the lungs following the infection challenge in frogs previously exposed to a pesticide mixture at different concentra-

tions (0.1×, 1.0×, 10×) or to control solutions (H<sub>2</sub>O, DMSO). For abbreviations, see Table 1

Treatment	Time to detection of <i>R. ranae</i> larvae in feces (days)			Prevalence of lung infection at day 21 post-challenge (%)
	Mean	SE	Range	
H <sub>2</sub> O	17.90	1.08	13–21	80
DMSO	18.10	1.05	13–21	70
0.1×	18.00	1.09	12–21	80
1.0×	14.90	0.64	13–19	100
10×	15.00	0.45	13–17	100



**Fig. 3** Mean abundance of the parasitic nematode *Rhabdias ranae* 21-day post infection challenge in frogs previously exposed to three dilutions of a pesticide mixture (0.1×, 1.0×, 10×) or to control solutions [water, dimethyl sulfoxide (DMSO)]. Values are presented as mean and SE. *Black bars* correspond to adult *R. ranae* recovered from the lungs only, whereas *gray bars* refer to the total number of nematodes including subadults and adults found in the peritoneal cavity plus adults established in the lungs. Results of the Ryan-Einot-Gabriel-Welsch multiple-range test are indicated by the *lowercase* and *uppercase* letters above the bars. Within each subgroup (lungs, total), bars having *no letter in common* are significantly different from one another

least one gravid *R. ranae* in their lungs, whereas no nematode had yet completed their migration to the lungs in 20–30% of the frogs in the other groups (water, DMSO and 0.1×). However, among-group difference in the prevalence of lung infection was not significant (Fisher's exact test,  $P=0.2304$ ).

At the end of the experiment, all frogs exposed to *R. ranae* larvae were infected by the parasite. No nematode other than *R. ranae* was found in the frog tissues confirming that the infection method used prevented multi-species infection. The total number of *R. ranae*, which is the sum of subadults in the peritoneal cavity and gravid adults in the lungs, was very similar among treatment groups (ANOVA,  $F_{4,42}=0.18$ ,  $P=0.9488$ ) and corresponded to almost 75% of the initial dose (Fig. 3). Two of the treated frogs from the 1× and 10× groups were found to be infected by 30 *R. ranae* (100% of the initial dose) meaning that all infective larvae to which they were

exposed penetrated the skin and survived in their body for up to 21 days post challenge. The mean number of adult *R. ranae* established in the lungs 21 days p.i. tended to increase with pesticide dosage (Fig. 3). On average, the lungs of 10×-exposed frogs were infected by twice as many worms as those of water-exposed frogs. Up to 13 adult nematodes were discovered in the lungs of frogs treated with the highest concentrations of pesticides (1× and 10×) whereas a maximum of eight worms had successfully migrated to the lungs in the water- and DMSO-exposed frogs. A significant effect of the treatment on mean abundance of gravid worms was detected (ANOVA,  $F_{4,45}=2.96$ ,  $P=0.0298$ ) but a Ryan-Einot-Gabriel-Welsch multiple-range test showed that only the 10×-exposed and the water-exposed groups were different from each other. We used a contrast analysis to perform a comparison among combined groups. This analysis revealed that the number of nematodes in the lungs was significantly higher ( $F_{1,45}=6.40$ ,  $P=0.0150$ ) in animals exposed to the two highest concentrations of pesticides (1× and 10×) when compared to the frogs exposed to the two control solutions (water and DMSO). Results of an ANCOVA ( $F_{7,42}=1.93$ ,  $P=0.0990$ ) performed to test for potential confounding effects indicate that the abundance of nematodes in the lungs was not significantly influenced by the sex of the host ( $F_{1,42}=0.05$ ,  $P=0.8203$ ), its length ( $F_{1,42}=0.44$ ,  $P=0.5098$ ) or weight ( $F_{1,42}=1.13$ ,  $P=0.2936$ ).

Mean length ( $\pm$ SE) reached by *R. ranae* 21 days p.i. in frogs exposed to water, DMSO, 0.1×, 1.0× and 10× mixture was respectively 6.33 mm ( $\pm 0.19$ ), 6.41 mm ( $\pm 0.16$ ), 6.44 mm ( $\pm 0.26$ ), 6.48 mm ( $\pm 0.17$ ) and 6.26 mm ( $\pm 0.22$ ). Neither the treatment to which frogs were exposed ( $F_{4,32}=0.21$ ,  $P=0.9287$ ), nor the number of *R. ranae* established in the lungs ( $F_{1,32}=0.69$ ,  $P=0.4127$ ) significantly affected worm size (ANCOVA,  $F_{9,32}=0.67$ ,  $P=0.7303$ ). This shows that the intensity of infection reached in this experiment was below a threshold at which intraspecific competition for space and food occurs.

## Discussion

In this study, juvenile leopard frogs were exposed to pesticides and then challenged with a parasitic nematode, *R. ranae*, in order to determine if agricultural contami-

nants can alter the infection pattern and modify the interaction between frogs and a parasite commonly encountered in the wild. The most significant result of this experiment was that the migration of *R. ranae* was accelerated in those hosts exposed to the highest concentrations of pesticides, which led to the accumulation of a greater number of adult worms in the lungs 21 days p.i. In addition, the prepatent period was shortened, meaning that development of the parasite was accelerated allowing an earlier onset of first reproduction. There was no difference in the size of the nematodes among treatments, implying that growth rates were similar at 21 days p.i. Given that fecundity is directly proportional to size in *Rhabdias* sp. (Goater 1992) and in other parasitic nematodes (Read and Skorpung 1995; Morand 1996; Marcogliese 1997), those worms that migrated faster probably did not suffer any loss of fecundity. This is the first study we are aware of that demonstrates alterations in basic parasite life history characteristics as a result of host exposure to contaminants. It remains to be seen how exposure to pesticides affects other life history traits and long-term parasite-host population interactions.

The actual mechanism leading to these changes is unknown, but presumably pesticides in the mixture interfered with the immune system, thus altering the frog's response to infection. The immune response to helminth infections tends to reduce or interfere with the establishment, survival and fecundity of parasites (Viney 2002a). For example, parasites are more fecund in immunodeficient animals (Viney 2002b). These generalizations are supported by observations in amphibians, whereby regulation of worm abundance occurs after infection due either to density-dependent effects or immune responses of the host (Tinsley 1995). In our study, the total number of worms recovered in frog tissues at the end of the experiment corresponded to 75% of the initial dose and was similar among treatment groups, showing that the skin of naïve leopard frogs is very susceptible to *R. ranae* larvae as observed in other anurans (Goater and Ward 1992) and that pesticide exposure did not influence host recognition and penetration components of the transmission process. Toxic interference with the skin defense system, such as the release of antimicrobial peptides (Maloy and Kari 1995), is thus unlikely to explain our results. Rather, differences among treatment groups in parasite life history characteristics occurred post-invasion. Findings of a companion study suggest that these differences result from modulation of certain components of the immune system. The most striking observation was a marked suppression of T lymphocyte proliferation in response to mitogens after a 21-day treatment with the pesticide mixture (Christin et al., in press). This decrease was observed in frogs exposed to the lowest concentration tested (0.1×) which correspond, for most pesticides in the mixture, to background levels typically detected in surface water of rivers draining agricultural areas (Fig. 1) (Giroux 1999). Moreover, this effect was found to persist up to 3 weeks after removal of pesticides in the 10×-exposed frogs (Christin

et al., in press). Similarly, depression of lymphocyte proliferation was detected in guinea pigs exposed to heavy metal toxicants, resulting in an enhanced abundance of migrating *Ascaris suum* in the lungs (Boroskova et al. 1995; Boroskova and Dvoroznakova 1997; Soltys et al. 1997). It is well established that lymphocytes play a determining role in the generation of an effective immune response against helminth parasites (Riffkin et al. 1996; Schallig 2000). Studies in mammals have shown that the susceptibility to intestinal nematodes was lowered in hosts exhibiting a high antigen-induced lymphocyte response, and this even occurred in naïve animals exposed for the first time to the parasite (Torgensen and Llyod 1992, 1993; Schallig 2000). Pesticide-induced alteration of lymphocyte functions, as observed by our team and other workers (Hoole 1997), could therefore affect innate immunity against parasitic helminths, in addition to the impact on acquired immune response mounted by animals exposed for a second time to a parasite. Thus, results herein, together with those of Christin et al. (in press), suggest that the immune system does indeed play a role in the migration, development and maturation of *R. ranae*. Alternatively, the parasite itself could have accelerated its migration as a direct response to contaminants or altered host physiology within highly exposed hosts, but this hypothesis appears less probable considering the immune suppression that was observed (Christin et al., in press). Post-invasion regulation of parasite populations may also result from density-dependent effects (Tinsley 1995). Given that the total number of worms recovered in frog tissues at the end of the experiment was not significantly different among treatment groups, any effect of density-dependence can be ruled out.

The ability of contaminants to increase the susceptibility of amphibians to pathogens has been frequently implied (Carey et al. 1999), but rarely scientifically demonstrated. Data on the effect of xenobiotics on host-parasite relationship are available for other animal taxa (e.g. Boroskova et al. 1995; Carlson et al. 2002), but to our knowledge, only two other studies addressed this issue in anurans. Taylor et al. (1999) exposed adult Woodhouse's toads (*Bufo woodhousi*) to sublethal doses of malathion (organophosphorus pesticide) before injecting them with *Aeromonas hydrophila*, a bacterium associated with red-leg disease in amphibians. Toads contaminated with malathion developed clinical disease and died at a significantly higher rate than control animals, clearly demonstrating that the virulence of this bacterium can be significantly increased by exposure of the host to insecticides. Such potentiating effect of pollutants on parasite pathogenicity was also observed by Kiesecker (2002). Combining laboratory exposures with field manipulations, he showed that stress in the form of pesticide exposure decreased the wood frog's (*Rana sylvatica*) ability to resist infection by common parasitic trematodes. While cercariae of *Ribeiroia* sp. and *Telorchis* sp. were equally successful in penetrating the skin of *R. sylvatica* tadpoles reared in either polluted or clean water, parasites were encysted in significantly

higher numbers in those animals previously exposed to atrazine, malathion or esfenvalerate (pyrethroid insecticide). Moreover, frog larvae exposed to agricultural runoff in the field were found to develop trematode-mediated limb deformities at higher rates than those growing in pristine ponds.

The influence of host exposure to pesticides on the pathogenicity of *R. ranae* was not specifically evaluated in this study. However, research using other host-parasite system has shown that life history characteristics related to parasite transmission are intimately linked with virulence; those parasites that are transmitted faster presumably become more virulent (Garnick 1992; Bull 1994; Ebert and Herre 1996; Frank 1996; Day 2001). In fact, while most scientists define virulence as the extent of damage or death caused to a host (Ewald 1994), others have observed that virulence is a property of both host and parasite (Bull 1994; Poulin and Combes 1999). Thus, Garnick (1992) referred to virulence as the ability to develop and reproduce in a host, and Poulin and Combes (1999) remarked that studies of virulence must emphasize the ability of the parasite to multiply. Given that we observed faster development, with earlier reproduction, and presumably no loss of fecundity, it can be surmised that parasites infecting frogs exposed to the higher concentrations of pesticides developed increased virulence.

Parasites of the genus *Rhabdias* are not considered highly virulent pathogens in adult amphibians. However, lungworm infection may have detrimental effects, especially in young anurans. The negative impact of a related species of nematode, *Rhabdias bufonis*, has been the subject of a series of investigations (Goater and Ward 1992; Goater et al. 1993; Goater 1994). Under certain conditions, lungworm infection of juvenile toads (*Bufo bufo*) negatively affected host growth and survival in a dose-related manner (Goater and Ward 1992). In addition, infection by *R. bufonis* at levels comparable to those found in our 10x-exposed frogs were shown to impair the ability of hosts to perform sustained physical activities, an effect thought to be partly attributable to interference of nematodes with the normal functioning of the lungs (Goater et al. 1993). Although possibly less deleterious than *R. bufonis* due to its smaller size (Goater and Vandenbos 1997), *R. ranae* probably affects its ranid host in a similar manner. An increased speed of migration in immunocompromised frogs as observed in this study could exacerbate the pathological effects of the parasite, with potentially serious ramifications for survival and fitness of frogs exposed to both toxicants and parasites. For instance, respiratory distress induced by lungworm infection could be critical for young-of-the-year frogs undertaking fall migration toward hibernation sites. In the northern part of the species range, metamorphs of *R. pipiens* do not emerge from reproduction ponds until early July, which leaves them limited time to grow up and store the fat reserves they need in preparation for hibernation (Wagner 1997). Under these constraints, an increased load of lungworms resulting from accelerated tissue

migration might also compromise the chances of young-of-the-year to survive several months of starvation. More research is needed to substantiate these hypotheses and determine if and how exposure of frogs to contaminants can actually increase lungworm virulence. Further studies should go beyond the traditional host-centered approach and also consider the response of the parasite to toxicants. Toxic substances leaching from agricultural lands — some having nematicidal properties — could impair the development and survival of free-living stages of *R. ranae* and/or negatively affect their infectivity.

Many authors have suggested the emergence of new highly virulent pathogens as an explanation for the disease-related die-off of amphibians in North America and Australia (Laurance et al. 1996; Daszak et al. 1999). Although several of these events have occurred in what appear to be relatively undisturbed environments (Carey et al. 1999), the role of immuno-modulating factors of natural or anthropogenic origin should be examined seriously. Environmental contaminants capable of interfering with the immune system have the potential to affect the host-parasite interaction, rapidly transforming a relatively harmless parasite into a virulent one. Our study contributes to this debate by showing that exposure to agricultural chemicals can alter the life history characteristics and infection dynamics of a common amphibian parasite, potentially increasing its virulence. Given the ubiquitous distribution of helminth parasites among animals in nature, results herein also have implications for all organisms exposed to pesticides or other contaminants in the environment.

**Acknowledgements** Lucie Ménard, Alain Branchaud and Stéphanie Gagné are acknowledged for their assistance in the laboratory and with frog rearing. We are also grateful to Dr Cameron Goater for sharing his expertise with culturing of lungworms and experimental infection of frogs. The image-analysing system of the Université de Montréal was used with permission from Bernadette Pinel-Aloul and technical assistance of Louise Pelletier. We thank Drs Cameron Goater and Robert Poulin, as well as two anonymous reviewers, for commenting on the manuscript. This project received the financial support of the Toxic Substances Research Initiative program (grant no. 46).

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