LETTERS

Agrochemicals increase trematode infections in a declining amphibian species

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Global amphibian declines have often been attributed to disease[1,2], but ignorance of the relative importance and mode of action of potential drivers of infection has made it difficult to develop effective remediation. In a field study, here we show that the widely used herbicide, atrazine, was the best predictor (out of more than 240 plausible candidates) of the abundance of larval trematodes (parasitic flatworms) in the declining northern leopard frog *Rana pipiens*. The effects of atrazine were consistent across trematode taxa. The combination of atrazine and phosphate—principal agrochemicals in global corn and sorghum production—accounted for 74% of the variation in the abundance of these often debilitating larval trematodes (atrazine alone accounted for 51%). Analysis of field data supported a causal mechanism whereby both agrochemicals increase exposure and susceptibility to larval trematodes by augmenting snail intermediate hosts and suppressing amphibian immunity. A mesocosm experiment demonstrated that, relative to control tanks, atrazine tanks had immunosuppressed tadpoles, had significantly more attached algae and snails, and had tadpoles with elevated trematode loads, further supporting a causal relationship between atrazine and elevated trematode infections in amphibians. These results raise concerns about the role of atrazine and phosphate in amphibian declines, and illustrate the value of quantifying the relative importance of several possible drivers of disease risk while determining the mechanisms by which they facilitate disease emergence.

There is growing appreciation of the importance of infectious diseases in driving population dynamics and declines of wildlife species[3,4]. However, investigators rarely assess the relative importance of the multitude of potential drivers and demonstrate a causal relationship between these factors and emerging infections so that the most problematic driver(s) could be effectively managed to prevent diseases of conservation concern[5]. Identifying the main risk factors for disease in amphibians is especially important because their widespread population declines have often been attributed to infectious disease[1,2]. Trematode infections of amphibians have attracted research and public attention predominantly because they can cause grotesque limb malformations, kidney damage and debility[6,7]. Furthermore, certain trematode infections are considered to be emerging diseases of amphibians driven by anthropogenic factors[7–10], and many can be lethal with the probability of amphibian mortality increasing with trematode load[6,11,12].

To understand the relative importance of drivers of amphibian trematode infections, we studied 18 wetlands in Minnesota, USA (Supplementary Table 1 and Supplementary Fig. 1), in which we measured more than 240 plausible predictors of amphibian trematode infections (Supplementary Table 2). We quantified larval trematode abundance in northern leopard frogs (*R. pipiens*; Supplementary Fig. 2), a species in decline across much of its range[13], and determined which of the factors best predicted the abundance of these parasites in these amphibians. The potential predictors we quantified included (1) the abundance of intermediate hosts of trematodes; (2) water quality and pollutants (Supplementary Table 3), which might suppress host immunity or be directly lethal to hosts or free-living stages of trematodes[14]; and (3) landscape attributes that might be crucial for supporting amphibians and avian and mammalian definitive hosts.

Our analyses showed that atrazine, the second most commonly used pesticide in the United States[15] and perhaps the world, combined with one of its metabolites, desethylatrazine (both from water samples), was the best predictor of total larval trematode abundance in *R. pipiens*. The sum of atrazine and desethylatrazine accounted for more variation than any other single predictor (51%, Monte Carlo randomization test, $P = 0.001$, Fig. 1a and Supplementary Fig. 3) and occurred in more models than any other predictor in a best subset analysis (Supplementary Table 4). For subsequent analyses, we focused on total larval trematode abundance because of the similar responses of larval trematode taxa to atrazine (Supplementary Table 5), and on atrazine and desethylatrazine combined because the combination was a better predictor of larval trematode loads than either variable alone and because the two compounds have similar half-lives and toxicity[16]. Hereafter, for brevity, we refer to the sum of detectable atrazine and desethylatrazine concentrations in water as 'atrazine'.

We proposed that atrazine augmented the richness and abundance of gastropods, the first intermediate host of trematodes, increasing the abundance of free-living trematode cercariae and the consequent exposure of *R. pipiens* to trematodes (Supplementary Fig. 4)[10]. This hypothesis was on the basis of the observation that atrazine and other herbicides regularly cause net increases in periphytic algae[17], a food source for many gastropods, despite herbicides being directly toxic to many periphyton species (see Supplementary Methods for mechanisms). In addition, we also suggested that atrazine increased the susceptibility of *R. pipiens* to trematode infections (Supplementary Fig. 4). This hypothesis was on the basis of laboratory studies which showed that low atrazine concentrations caused immunosuppression in ranid frog species[18,19] and an ambystomatid salamander[20], increasing larval trematode loads and susceptibility to viral infections, respectively[19,20].

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We used a path analysis to explore the amount of support for these proposed mechanisms for how atrazine might increase larval trematode loads[21]. Despite the low sample size of the path analysis, almost every path was significant (Fig. 1b) and the parameters were generally robust to bootstrapping. Parameter significance was also unaffected when controlling for the relationship between atrazine and phosphate (Supplementary Table 6), the latter of which is a primary ingredient in fertilizers that can also increase larval trematode loads[10]. The path model showed that atrazine was positively related to gastropod abundance and richness, and that gastropod richness was positively associated with larval trematode loads (Fig. 1b). Independent of this exposure-mediated effect on infection risk was a significant positive association between atrazine and larval trematode abundance (Fig. 1b), consistent with atrazine increasing the susceptibility of *R. pipiens* to trematodes.

To test this susceptibility hypothesis, we quantified melanomacrophage aggregates in the livers of frogs from each wetland (Supplementary Fig. 5). Melanomacrophages are cells of macrophage lineage (that is, Kupffer cells) found in the kidneys, spleen and liver that seem to be involved in inflammatory responses, in responding to foreign and dead material, and in fighting infections by a variety of parasites including encysted larval helminths[22–24]; furthermore, they have been proposed as reliable biomarkers for water pollution[24]. We did not incorporate melanomacrophage aggregates into the path analysis because, unlike the other variables in the model (Fig. 1b), melanomacrophage aggregates seemed to be significantly increased by the richness and abundance of nematodes and adult trematodes that also infected the frogs (Supplementary Table 7). Consequently, we controlled for these co-infections when testing for relationships between melanomacrophage aggregates and atrazine, and between melanomacrophage aggregates and larval trematode loads. Hereafter, nematode- and adult trematode-independent melanomacrophage aggregate levels will be referred to as melanomacrophage aggregate scores.

We discovered that atrazine was a significant negative predictor of melanomacrophage aggregate scores in *R. pipiens* (Fig. 2a). Phosphate (in water samples) was also a significant negative predictor of melanomacrophage aggregate scores (Fig. 2b). Similarly, in a laboratory study, both atrazine and fertilizer decreased peripheral leukocyte concentrations in tiger salamanders[20]. Moreover, the melanomacrophage aggregate score was a significant negative predictor of larval trematode abundance in *R. pipiens* (Fig. 2c), supporting the assertion that pollution-related immunosuppression was an important contributor to elevated larval trematode loads.

We found little evidence that atrazine was correlated with any other quantified variables that could parsimoniously explain the observed patterns; no variables that were correlated with atrazine and larval trematode loads were correlated with gastropod richness and melanomacrophage aggregate scores (Supplementary Table 8). Specifically, water column phosphate—which also can elevate amphibian trematode loads by increasing periphytic algae and gastropod abundance[10]—was not significantly correlated with atrazine and, when tested alone, was not a significant predictor of the abundance of larval trematodes in *R. pipiens* (Supplementary Table 8). However, when we included phosphate and atrazine in our statistical model, both variables were significant positive predictors of larval trematode burdens in *R. pipiens* ($F_{1,14} = 12.17$, $P = 0.004$, and $F_{1,14} = 31.06$, $P < 0.001$, respectively), indicating that they accounted for relatively uncorrelated components of variation in larval trematode loads in the study region. Moreover, the combination of atrazine and phosphate accounted for 74% of the variation in larval trematode abundance ($F_{2,14} = 19.75$, $P < 0.001$), and atrazine and phosphate were in 19 and 17 of the top 20 best subset models for larval trematode abundance, respectively (Supplementary Table 4).

We conducted a community-level mesocosm experiment to test whether the detected correlation between atrazine and amphibian trematode loads was indeed causal. Our mesocosm experiment showed that, in just one generation, cattle tanks with a single dose of atrazine at an expected environmental concentration had more than four times as many snails as control tanks (Table 1). Atrazine significantly reduced phytoplankton abundance, which resulted in significantly increased nutrient availability, water clarity and sunlight penetration to attached algae (Table 1). Tanks with atrazine had significantly greater periphyton levels than control tanks (Table 1), which presumably fuelled the elevated snail reproduction and perhaps increased snail richness in ponds with elevated atrazine (due to productivity–diversity relationships; Supplementary Methods).

As in the field survey, there was a significant association between atrazine exposure and elevated frog susceptibility in our mesocosm experiment. The mesocosm experiment was conducted in the southern range of *R. pipiens* where this species is declining[13]. Consequently, we conducted our mesocosm experiment on two congeners, *Rana palustris* (pickerel frog) and *Rana clamitans* (green frog). *Rana palustris* had significantly lower liver melanomacrophage counts in atrazine tanks compared to control tanks (Table 1), and a similar trend was observed for their liver eosinophils (Table 1), another important immune cell for fighting larval trematodes[19]. *Rana palustris* survival

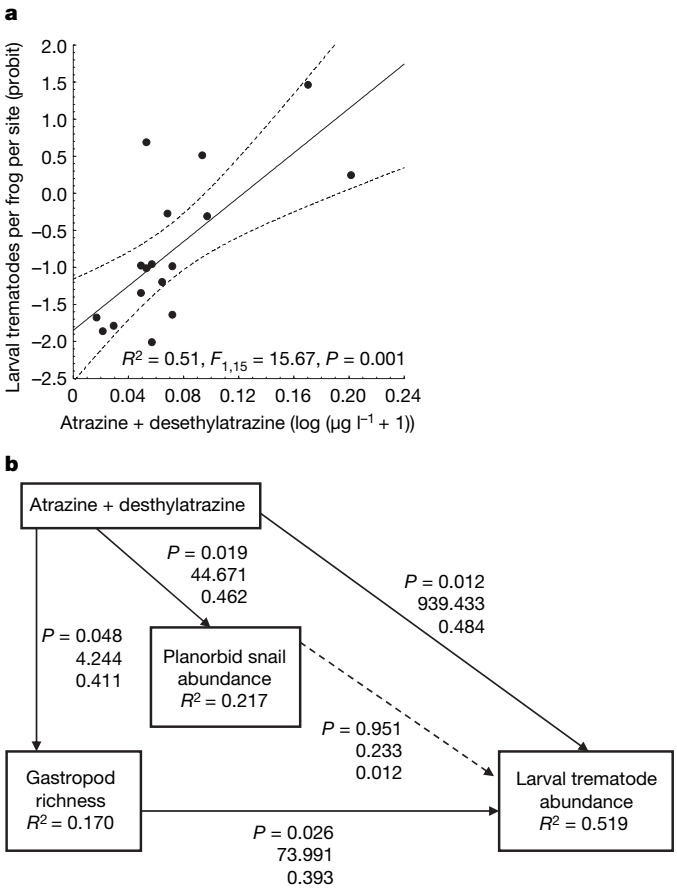

Figure 1 | Proposed mechanisms for the relationship between atrazine and larval trematodes in amphibians. a, The relationship between detectable concentrations of atrazine plus desethylatrazine in water samples and larval trematode abundance ('probit' transformed, that is, the normal standard deviate of mean abundance/1,000) in northern leopard frogs, *R. pipiens*. Each data point represents a wetland, and a 95% confidence band is shown. **b**, Results of a path analysis examining the suggested mechanisms by which atrazine plus desethlyatrazine increase larval trematode abundance. Unstandardized and standardized coefficients, respectively, are provided next to each path. Solid arrows represent significant paths. The model chi-square was 0.339 (d.f. = 1, $P = 0.561$), indicating that the model was a good fit to the data.

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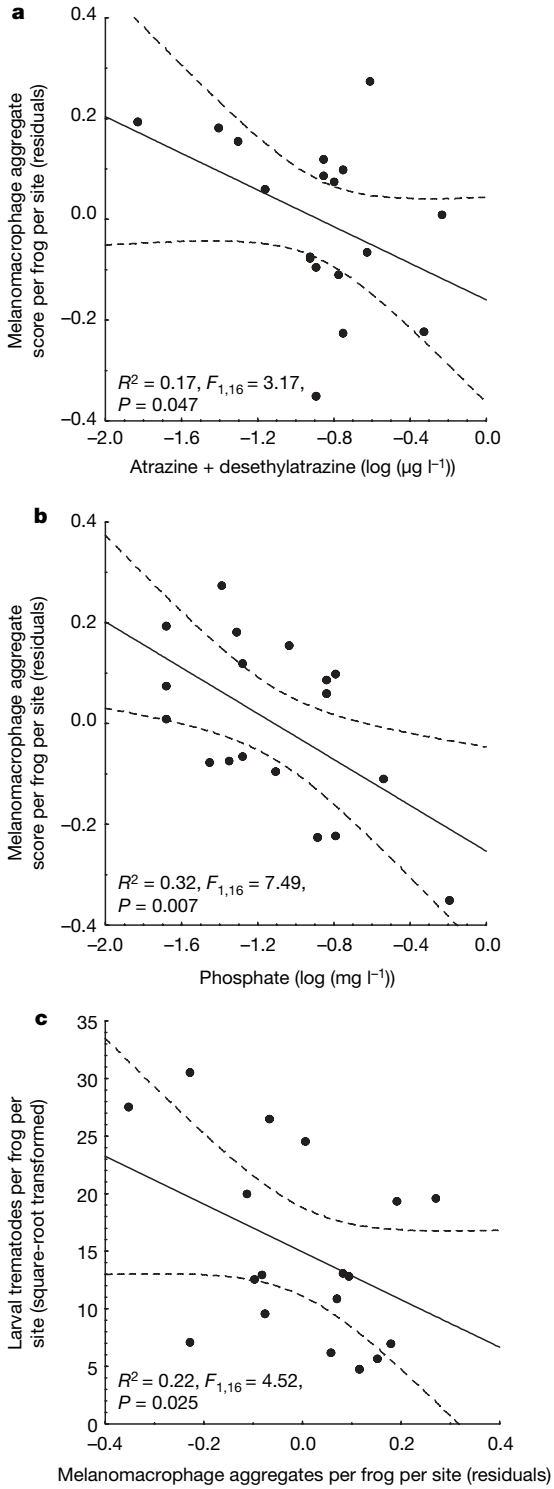

Figure 2 | Relationships between melanomacrophage aggregates and atrazine, phosphate and larval trematode loads. **a**, The relationship between melanomacrophage aggregate score in *R. pipiens* and concentrations of atrazine plus desethylatrazine in water samples. **b**, The relationship between melanomacrophage aggregate score in *R. pipiens* and phosphate concentration in water samples. **c**, The relationship between melanomacrophage aggregate score in *R. pipiens* and larval trematode abundance. In these graphs and analyses, we used the residuals from the relationship among mean melanomacrophage aggregate scores and the richness and abundance of nematodes and adult trematodes in leopard frogs (see text). Each data point represents a wetland, and 95% confidence bands are shown.

was significantly lower in the tanks with atrazine than in the tanks without atrazine (Table 1), preventing us from conducting an unconfounded test of the effect of atrazine on trematode loads in this species because it is probable that the most highly infected individuals were those that died.

For *R. clamitans*, atrazine exposure was not significantly associated with reduced liver melanomacrophages or elevated mortality, but it was associated with significant reductions in liver eosinophil counts (Table 1). Hence, in both ranid species, atrazine significantly suppressed immune parameters known to be important for fighting larval trematodes[19,22–24], a result consistent with previously documented immunosuppression in *R. pipiens* and *Rana sylvatica* exposed to atrazine[18,19]. Furthermore, in atrazine tanks, *R. clamitans* had significantly more plagiorchid trematodes than in control tanks when controlling for the relationship between plagiorchid loads and eosinophils (Table 1), suggesting that atrazine had effects on trematode infections that were independent of its effects on eosinophils (Supplementary Methods).

Larval trematode loads in amphibians must depend on the abundance of definitive hosts, such as amphibian-eating birds, because they are the source of trematode eggs in wetlands; thus, an important caveat is that atrazine and phosphates cannot increase trematode infections unless there is an ample supply of trematodes to begin with. This dependence is supported by the significant interaction between atrazine and the number of habitat patches which were 'suitable' for definitive hosts (forest, wetlands and open water) around each wetland (Monte Carlo randomization test: 1 km radius, $P = 0.004$; 10 km radius, $P = 0.019$), the latter of which is a proxy for visitations by, and for the abundance of, definitive hosts. Knowledge of this context-dependency should facilitate appropriately targeting remediation strategies for amphibian trematode infections.

Our findings suggest that atrazine, at concentrations commonly occurring in freshwater ecosystems, can be a primary driver of larval trematode infections for a declining amphibian species, and that phosphate-associated eutrophication has an important, and perhaps complementary, role (because it was only significant when atrazine was included as a covariate). Both atrazine and phosphate appear to increase exposure and susceptibility to larval trematodes by augmenting snail intermediate hosts[10] and by suppressing amphibian immune responses. By evaluating more than 240 alternative hypotheses, a number which could never realistically be examined in a manipulative experiment, and by demonstrating consistent results and underlying mechanisms between our controlled mesocosm experiment and wetland survey, we have substantially reduced the probability that the relationships identified are spurious. Hence, we provide support for the hypothesis that reducing atrazine and phosphate inputs to wetlands might effectively remediate elevated and often debilitating amphibian trematode infections.

Notably, the potentially important nexus between amphibian parasitism and pollution demonstrated here would not have been detected in standard studies used to register chemicals in the United States and Europe because these studies are typically conducted on individuals isolated from other species with which they naturally coexist, such as their parasites[25,26]. Therefore, greater effort is needed to understand the potentially important effects of pollution on species interactions that may contribute to reduced host survival[14,26]. Additionally, the results of this study—along with other studies linking pesticide use with amphibian declines[27] and linking low levels of atrazine with endocrine disruption, hermaphroditism[28] and mortality in amphibians[29,30]—raise serious concerns about the relationship between atrazine and global amphibian losses. Altogether, our work demonstrates the value of quantifying the relative importance of several plausible drivers of disease risk and population declines using a combination of field surveys and manipulative studies to enable rational and prioritized environmental remediation.

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Table 1 | Findings from mesocosm experiment examining the effect of atrazine on factors proposed to influence larval trematode loads in frogs.

Response variable	Atrazine ($n = 4$)		Control ($n = 8$)*		Test statistic	P†
	Mean	s.e.m.	Mean	s.e.m.		
Snail hatchlings m^{-2}	468.09	205.85	110.64	29.31	20.19‡	<0.001
Snail egg masses m^{-2}	30.45	3.03	12.37	4.34	4.98‡	0.026
Chlorophyll *a* in phytoplankton ($\mu g\, l^{-1}$)	2.52	0.45	7.14	1.67	4.88§	0.031‖
Water clarity (scale 1–5)	4.75	0.25	3.88	0.23	5.25¶	0.011‖
Chlorophyll *a* in periphyton ($mg\, m^{-2}$)	1.04	0.18	0.70	0.08	7.17§	0.032
Melanomacrophages per field of view for *R. palustris* (log)	0.16	0.06	0.36	0.06	4.50§	0.031‖
Eosinophils per field of view for *R. palustris* (log)	0.03	0.14	0.21	0.06	1.88§	0.102‖#
Survival of *R. palustris* (%)	47.50	7.50	70.00	3.90	9.77§	0.017
Melanopmacrophages per field of view for *R. clamitans* (log)	0.25	0.13	0.38	0.09	0.67§	0.217‖*
Eosinophils per field of view for *R. clamitans* (log)	0.11	0.10	0.39	0.07	4.75§	0.029‖
Survival of *R. clamitans* (%)	76.25	3.15	76.25	2.80	<0.001§	0.971**
No. of plagiorchid trematode cysts in *R. clamitans*	20.61	4.52	7.51	3.30	4.90§	0.031‖††

* The solvent and water controls were pooled because there was no significant difference between these treatments.
† Block was included in the model if its P value was less than 0.25.
‡ χ^2 statistic from analyses using the generalized linear model with a Poisson error distribution and a log link.
§ F statistic from analyses using the general linear model.
‖ One-tailed test.
¶ χ^2 statistic from analyses using the generalized linear model with an ordinal multinomial error distribution and a logit link.
#The power of the test was 0.646; the minimum sample size necessary per group to detect a significant difference given the power was six.
* The power of the test was 0.326.
** The power of test was 0.056.
†† Controlling for biomass of plagiorchid-infected snails ($F_{1,7} = 5.45$, $P = 0.026$) and mean log eosinophil counts in tadpoles ($F_{1,7} = 3.58$, $P = 0.050$).

METHODS SUMMARY

In each wetland in 1999, we characterized biological diversity (three visits: March–April, May–June and July–August; Supplementary Tables 2 and 3) and obtained water, sediment and amphibian tissue samples to quantify various analytes (two visits: April–May and June–July; Supplementary Table 3). Analyte quantification was conducted by the Illinois Waste Management and Research Center using standard US Environmental Protection Agency protocols. During wetland visits, we attempted to collect 15 recently metamorphosed *R. pipiens* for parasite quantification and 25 for pathology.

Our mesocosm experiment was conducted at Pennsylvania State University using a randomized block design and twelve 1,100-litre plastic cattle tanks (800 l of water) covered with shade cloth. Three weeks before dosing, each tank received 300 g of mixed hardwood leaves, plexiglass to sample snail egg masses and hatchlings, periphyton samplers and inoculations of zooplankton, periphyton and phytoplankton from four ponds. Just before dosing, each tank received four larval amphibian (5 *Ambystoma maculatum*, 20 *Hyla versicolour*, 20 *R. palustris* and 20 *R. clamitans*), two snail (11 *Planorbella trivolvis* and 10 *Physa gyrina*), one beetle (5 *Hydrochara* sp.), two water bug (2 *Belostoma flumineum* and 7 *Notonecta undulate*) and one dragonfly species (2 *Anax junius*). We applied a single dose of technical grade atrazine at 102 $\mu g\, l^{-1}$, an expected environmental concentration (mean actual concentration was 117 $\mu g\, l^{-1}$). The experiment ran for 4 weeks, from the end of June to July 2007.

All variables were quantified blind to the wetland characteristics or mesocosm treatment. Wetlands and mesocosms were used as the sample units for statistical analyses. That is, if several measurements were taken on a wetland or a tank, statistical analyses were conducted on the mean of those measurements. The path analysis was conducted assuming linear relationships among the raw data and using maximum likelihood estimation and bootstrapping[21].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions For the field survey, V.R.B. and L.B.J. designed the data collection. C.M.J., P.K.S., C.L. and A.M.S. conducted the survey. C.M.J. coordinated data collection, assembly and management. M.D.P. conducted all analyte analyses. A.M.S. performed amphibian necropsies of *R. pipiens* for parasite quantification. C.L. quantified amphibian immunity. For the mesocosm study, J.R.R., T.R.R. and J.T.H. designed and implemented the experiment. J.R.R. oversaw all components the study. T.R.R. and N.H. processed amphibian samples and quantified amphibian immune parameters. H.J.C. quantified periphyton and phytoplankton. J.R.R. conducted all statistical analyses and wrote the paper. A.M.S. wrote parts of the Supplementary Methods. The paper was edited by all authors.

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METHODS

Wetland survey. Quantification of faunal diversity (amphibians and macroinvertebrates) was conducted using daytime visual time-constrained and area-constrained dip-net sampling methods, adjusting effort according to the size of the wetland. Three 10-m transects were randomly placed within each community type in and around each wetland, and the line-intercept method was used to record the relative cover of each plant species under or over the line.

Landscape structure—including landscape composition, fragmentation patterns, patch density and connectivity patterns—was quantified around each wetland using Fragstats (version 2). Land cover data were derived from the 1990s National Land Cover Database, which was based primarily on 1992 Landsat-5 Thematic Mapper data, with a 30-m resolution. These data were used to summarize landscapes at 1-km and 10-km extents. The 1-km extent was chosen because it represents the approximate dispersal capabilities of leopard frogs, and it allowed for exploration of effects of adjacent land use on the parasite communities. The 10-km scale was examined because this larger spatial scale might affect the highly mobile definitive hosts of many frog parasites (for example, birds and mammals).

The buccal and abdominal cavities were examined, and all visceral organs, skin and eyes were removed from necropsied frogs and inspected for parasites. After necropsy, the remaining frog carcasses were fixed in 10% neutral buffered formalin and cleared and stained. Encysted metacercariae in the musculature were identified and enumerated by examining the cleared and stained specimens under a dissecting microscope (Supplementary Fig. 2).

Frog livers were embedded in paraffin, stained with haematoxylin and eosin, sectioned and then examined under a light microscope. Melanomacrophage aggregate numbers were scored from zero to five, in which zero indicated that no melanomacrophage aggregates were present and five indicated that more than 90% of the liver was filled with melanomacrophage aggregates (Supplementary Fig. 5). Livers that fell in between categories were assigned intermediate values. All quantification was done on frogs of Gosner stage 46.

Our statistical analysis for the wetland survey was done in four steps. In the first step, we calculated the coefficient of determination (R^2) for each predictor in the absence of covariates to determine which single predictor accounted for the most variation in larval trematode abundance. In our second step, we used the general linear model to generate the 20 best subset models for mean larval trematode loads in *R. pipiens*. Models were ranked by adjusted R^2 values and were limited to a maximum of three predictors (Supplementary Table 4). In our third step, we used a structural equation model, which was later reduced to a path analysis on the basis of a confirmatory factor analysis (see Supplementary Methods and Supplementary Fig. 6), to evaluate the amount of support for the specific proposed causal mechanisms underlying the significant positive relationship between atrazine concentration and larval trematode abundance (Fig. 1b). Phosphate was not included in our path model because the primary emphasis was on atrazine and our sample size was too small to include both chemicals in the model. Path analysis can be sensitive to relatively small samples sizes, raising concerns about the precision of parameter estimates and the stability of our path model. To evaluate the robustness of the parameter estimates and model stability, we obtained standard error estimates for all the paths in Fig. 1b using both maximum likelihood and bootstrapping methods (200 resamplings)[21]. Another matter is that herbicides and fertilizers might positively co-vary in the landscape, raising concerns about the validity of the path model if the relationship between phosphate concentration and atrazine was not controlled. We re-calculated all the parameter estimates and standard errors controlling for the relationship between atrazine and phosphate concentrations. Structural equation modelling and path analysis and bootstrapping were done using SEPATH in Statistica 6.1 (StatSoft Inc.). The final step was to assess whether atrazine was correlated with any other variables that might more parsimoniously explain the observed patterns. We used correlation analysis to identify any variables that were positively correlated with atrazine, larval trematode loads, gastropod richness and melanomacrophage aggregate scores.

Mesocosm experiment. All of the species in the mesocosm experiment naturally coexist and were applied at densities found locally. The applied expected environmental concentration of atrazine was calculated using the US Environmental Protection Agency's GENEEC (version 2) software (Supplementary Methods).

All snails used in the experiment were screened twice for patent (shedding) trematode infections before being placed in the tanks. In addition to the 11 screened planorbid snails that were free to move in each tank, each tank also received a caged planorbid snail shedding cercariae from the family Plagiorchidae. These caged snails were rotated among the tanks every other day in an attempt to homogenize exposure to trematodes. Snails were thoroughly rinsed before being moved to another tank and the cages were not rotated.

At the end of the experiment, we took water samples for phytoplankton analyses, scraped periphyton from the periphyton samplers, scored water clarity on a scale from 1 to 5, and counted the snail egg masses on the walls of each tank, the snail hatchlings from the plexiglass placed in each tank and the surviving and dead animals. Chlorophyll *a* concentrations were measured using standard fluorometric techniques. Five preserved *R. clamitans* and five *R. palustris* tadpoles from each tank were selected randomly and melanomacrophage and eosinophils cells were counted per field of view from their sectioned and stained livers. Each tadpole was also cleared and stained and their larval trematodes were counted under a compound scope, as described previously.

We re-screened each snail from trematodes at the end of the experiment and discovered that some of the snails that were placed in the tanks were pre-patent (infected but not shedding treamtodes) and began shedding plagiorchid cercariae during the experiment. Consequently, all snails were weighed and dissected to ensure that we did not miss any infected snails that were not shedding at the end of the experiment. One tank had a snail infected with strigeid trematodes (determined on the basis of snail dissection and shedding) and we could not discriminate these trematode metacercariae from the plagiorchid metacercariae, and thus, this tank had to be dropped from the trematode load analyses.

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