BIOLOGY LETTERS

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Cite this article: Almeida RM, Han BA, Reisinger AJ, Kagemann C, Rosi EJ. 2018 High mortality in aquatic predators of mosquito larvae caused by exposure to insect repellent. *Biol. Lett.* **14**: 20180526. http://dx.doi.org/10.1098/rsbl.2018.0526

Received: 19 July 2018
Accepted: 4 October 2018

Subject Areas:
ecology, environmental science, health and disease and epidemiology

Keywords:
salamander, predation, arbovirus, socioecology, mosquito control, food web

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Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.4265120.

THE ROYAL SOCIETY PUBLISHING

Global change biology

High mortality in aquatic predators of mosquito larvae caused by exposure to insect repellent

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In the face of mosquito-borne disease outbreaks, effective mosquito control is a primary goal for public health. Insect repellents, containing active compounds such as DEET and picaridin, are a first defence against biting insects. Owing to widespread use and incomplete sewage treatment, these compounds are frequently detected in surface waters, but their effects on aquatic taxa such as mosquito larvae or their naturally occurring aquatic predators are poorly understood. We investigated the effects of environmentally realistic concentrations of commercial products containing DEET and picaridin on survivorship of mosquito larvae, and their potential indirect effects on survival of larval salamanders, a major predator of mosquito larvae. Larval mosquitos were not affected by exposure to repellents containing DEET or picaridin. We found no larval salamander mortality in control and DEET treatments, but mortality rates in picaridin treatments ranged from 45 to 65% after 25 days of exposure. Salamander larvae exposed to repellents containing picaridin began to display tail deformities and impaired development four days after the experiment began. Our findings suggest the possibility that environmentally realistic concentrations of picaridin-containing repellents in surface waters may increase the abundance of adult mosquitos owing to decreased predation pressure.

1. Background

Owing to the recent outbreaks of mosquito-borne viruses like Zika, dengue and chikungunya in the Americas, the use of insect repellents has increased dramatically. For instance, during the recent Zika virus epidemic in Brazil, sales of insect repellents increased by 115% in December 2015 (the peak month for infections) compared to the year before [1]. Repellent suppliers in the USA also saw skyrocketing demand for their products [2]. Since the 1960s, the two most widely used insect repellent formulations contain either the active ingredient DEET (*N*,*N*-diethyl-m-toluamide), or an alternative repellent containing the active ingredient picaridin (also known as icaridin, saltidin, Bayrepel and KBR 3023) [3,4].

The detection of active ingredients of insect repellents in natural environmental (such as national parks) and surface waters—particularly DEET [5]—has raised concern about how these compounds affect non-target species [6]. The main pathway for repellents containing these compounds to enter aquatic ecosystems is via sewage effluent. Since the late 1990s, when repellents containing DEET started to be replaced with repellents containing picaridin, picaridin has been detected in German wastewater influents, with concentrations of up to

3000 ng l^{-1} [4]. In wastewater effluents, however, concentrations were non-detectable, likely owing to rapid primary aerobic biodegradation [4,7]. While both DEET and picaridin have antimicrobial properties [8], the risks posed by DEET to aquatic biota are considered low [6]. The toxicity of picaridin to rainbow trout, zebrafish, green algae and water flea has been previously tested, but the endpoints are on the order of milligrams per litre for all tested species [9]. Two studies have investigated behavioural effects of environmentally realistic concentrations of picaridin on aquatic arthropods [10,11], but no effects were found. In particular, the impacts of these insect repellents on mosquito predators, such as amphibians, are not known. Amphibians are highly sensitive bioindicators of environmental pollution, and their global population declines have been linked to a growing list of manufactured fertilizers, insecticides and other contaminants [12]. The negative impacts of mosquito repellents on non-target species that regulate mosquito populations have the potential to outweigh the benefits of temporarily repelling bites from adult mosquitos.

Neither DEET nor picaridin is readily soluble, thus commercially available mosquito repellents that can be purchased by the typical consumer contain several other compounds that act in concert to make DEET and picaridin easier to apply topically. It is these more complex commercial formulations that end up in natural environments and surface waters. Here, we present a series of experiments to quantify the impacts of environmentally realistic concentrations of two commercially available insect repellent formulations containing DEET and picaridin. Specifically, we ask whether repellents containing DEET and picaridin directly affect (1) the survival of mosquito larvae and (2) early development of larval salamanders, which are natural predators of mosquito larvae. Given the efficiency with which salamanders consumed mosquito larvae in our experiments, and the high mortality observed in salamander larvae caused by exposure to the repellent containing picaridin, our results suggest that repellents containing picaridin may have potentially serious direct implications for ecological health.

2. Methods

We examined the sensitivity of mosquito larvae and larval salamanders to two commercial products: Repel 100 Insect Repellent (98.1% DEET based on the manufacturer's label) and Sawyer Premium Insect Repellent (20% picaridin based on the manufacturer's label). These are the two most concentrated forms of insect repellents that use DEET and picaridin available in the US market. Serial dilutions were prepared using distilled water and these two commercial products.

Spotted salamanders (*Ambystoma maculatum*) were collected as eggs from a vernal pond at the Cary Institute of Ecosystem Studies (Millbrook, NY, USA). Larvae hatched in a single 10-gallon aquarium representing a study population of two mixed clutches. Every other day, salamander larvae were fed a diet of *Daphnia* and other aquatic invertebrates collected from the natal pond. Experimental exposures were initiated when larval salamanders reached developmental stage 41 [13], when larvae are freely swimming, foraging, external gills and forelimb buds are visible. Larval salamander hatchlings were exposed to one of four concentrations of repellents containing DEET (target concentrations: 0, 98.1, 981 and 9810 ng DEET l^{-1}) or picaridin (target concentrations: 0, 20, 200 and 2000 ng picaridin l^{-1}) for 25 days. These concentrations were applied once at the beginning of the experiment, and were chosen to represent the reported range of DEET and picaridin concentrations in surface waters worldwide [4,6]. The concentrations in our experiments are conservative approximations because we prepared concentrations based on unadulterated commercial formulations, not the concentrations of pure active compounds. Each treatment consisted of three replicate chambers (1000 ml glass jars filled with corresponding volumes of the respective treatment added to aerated groundwater, without any substrate), and three salamanders were placed into each replicate chamber, totalling nine salamanders per treatment. Deformed tails (i.e. tails that are improperly formed during larval development and are permanently kinked or bent) are a common indicator of developmental abnormality in amphibian larvae [12,13]. Tail deformity and mortality were assessed approximately three times per week during the experiment. Developmental stage and body length were assessed on days 1, 4 and 12, when salamanders in the control treatment reached developmental stage 46 (three digits visible on each forelimb) [14]. To test for significant differences in mortality and tail deformity among treatments, we ran linear mixed effects models with mortality and tail deformity as the two response variables and insect repellent concentration as a fixed effect, and day since exposure as a random effect, using the *lmer* function of the package *lme4* [15]. We applied pairwise post hoc tests using the function *lsmeans* of the package *lsmeans*, followed by a Tukey HSD p-value adjustment [16]. All analyses were conducted in the R statistical environment, v. 3.3.2 [17]. For the statistical analyses, we used the cumulative mortality and tail deformity for each treatment chamber (three chambers per treatment).

We used a 50 µm plankton net to collect larvae of several mosquito species (*Aedes japonicus*, *Aedes triseriatus*, *Culex restuans*, *Culex territans*) from the same pond from which salamander larvae were collected. The mosquito larvae were immediately transported to the laboratory and 30 individuals were exposed to each of the repellent treatment concentrations of DEET (98, 980 and 9800 ng l^{-1}) and picaridin (20, 200 and 2000 ng l^{-1}) for 5 days. Mortality of mosquito larvae was assessed daily, and the experiment was stopped when mosquito larvae started to pupate.

A large body of literature shows that salamanders and other urodeles are important predators of mosquito larvae. To confirm this in our system, we also conducted a separate mesocosm study to confirm whether, and how efficiently, larvae of *A. maculatum* consume mosquito larvae (see electronic supplementary material, S1 for methods and results of this experiment).

3. Results

We confirmed that larval salamanders are voracious predators of larvae of various local mosquito species (electronic supplementary material, S1). Larval mosquitos were not affected by exposure to environmentally realistic concentrations of repellents containing DEET or picaridin. Mortality was registered for only one individual in the low-concentration DEET repellent treatment (data not shown). We observed no effect of DEET repellent treatments on salamander larvae (electronic supplementary material, S2), but mortality rates in picaridin repellent treatments ranged from 45 to 65% after 25 days of exposure (figure 1*d*). No mortality was observed in the control treatments (figure 1*d*). All picaridin repellent treatments exhibited higher tail deformity ($F = 59.0$, $p < 0.05$) and mortality ($F = 58.0$, $p < 0.05$) compared to the control treatment. Effects of picaridin repellent exposure on tail deformity, developmental stage and body

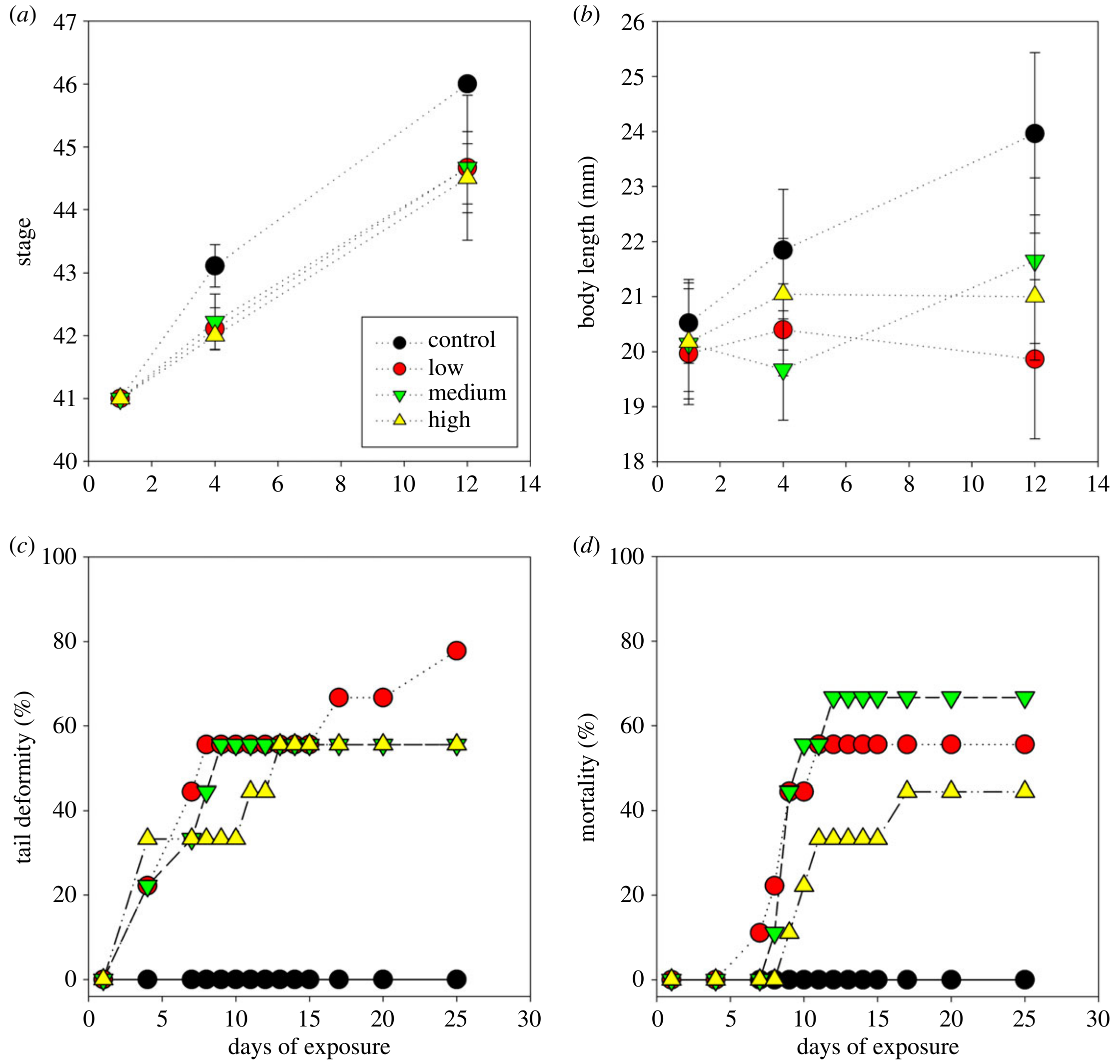

Figure 1. Effects of exposure to a picaridin-containing repellent on salamander (*a*) stage ($\pm$s.d.), (*b*) body length ($\pm$s.d.), (*c*) cumulative tail deformity, and (*d*) cumulative mortality. Low, medium, and high refer to treatments with 20, 200 and 2000 ng l^{-1}.

length began on day 4, with salamander larvae displaying less deformity, greater growth and development in the control treatment compared to picaridin repellent treatments (figure 1*a–c*). Notably, no mortality had been observed by day 4 in any experimental treatment (figure 1*d*), which is the maximum exposure time typically used in ecotoxicological studies to determine the lethal concentrations that kill 50% of tested individuals (LC50). Thus, in a typical LC50 toxicology test, the repellent containing picaridin would have been deemed non-toxic despite clear effects on mortality and deformity over a 25 day period.

4. Discussion

Our findings demonstrate that larval salamanders suffer severe mortality and developmental deformities owing to environmentally relevant exposures to a commercially available repellent containing the active ingredient picaridin. Our experiments also demonstrate that larval salamanders are an important predator of larval mosquitos, which appear to be unaffected by exposure to repellents containing DEET or picaridin. Ecosystems contaminated with picaridin repellent may be at risk of further declines in salamander populations.

Salamanders have been experiencing dramatic population declines globally in response to increasing pressure from anthropogenic stressors such as pollution, habitat loss and climate change [12,18]. These declines pose a great threat to ecosystem integrity [19]. Amphibians are key in connecting energy and matter between aquatic and terrestrial ecosystems, and they control ecosystem structure and function through predation [20,21]. Because salamanders are voracious predators of mosquito larvae during the aquatic phase, high mortality of salamander larvae caused by exposure to picaridin repellent suggests the possibility that more adult mosquitos may emerge from aquatic systems depauperate of salamander predators (figure 2). This feedback loop may be exacerbated in systems where exposure to picaridin repellent coincides with salamander breeding seasons that are much shorter compared to mosquitos'. Most mosquitos have an extended season of continuous breeding and are therefore better able to compensate for fluctuating predator populations, leading to more larvae emerging successfully as adults in the absence of population regulation by aquatic predators.

Our results suggest several important avenues for future study. First, controlled outdoor mesocosm studies would inform whether the high mortality rates we observed in a laboratory setting are also observed under semi-natural conditions that may degrade, adsorb or neutralize lethal

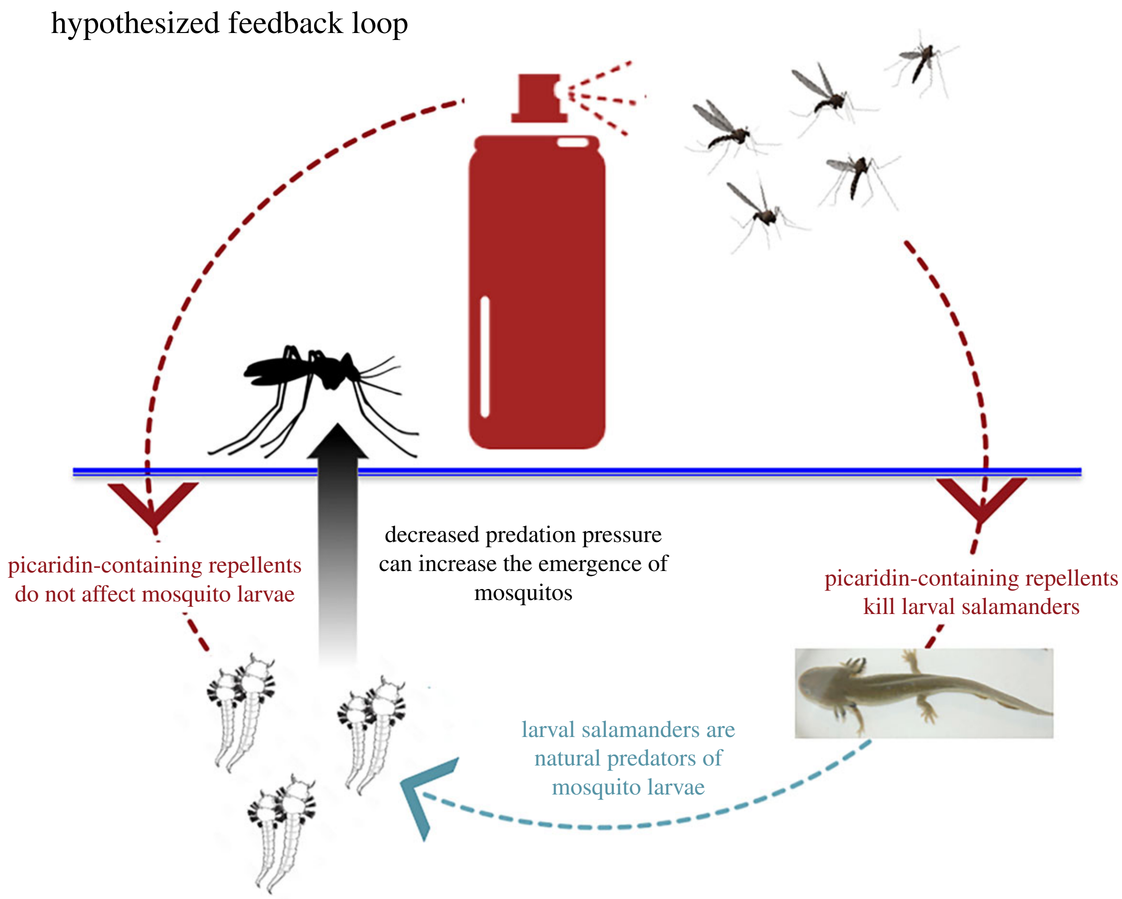

Figure 2. Hypothesized direct and indirect effects of picaridin on aquatic predators of mosquito larvae indicating a human–environment positive feedback loop. Picaridin-containing repellents are used by humans to repel insects, and may end up in aquatic ecosystems. The picaridin-containing repellent we tested did not directly affect mosquito larvae survival or development, but trace concentrations of it caused substantial mortality of larval salamanders under laboratory conditions. We hypothesize that a loss of predators may in turn increase the emergence of mosquitos in impacted ecosystems, which can possibly trigger increased application of picaridin repellent and thus lead to a positive feedback loop. (Online version in colour.)

compounds present in formulations of commercially available picaridin repellents. We exposed salamander larvae at the earliest developmental stages, thus future experiments on later developmental stages are necessary to understand the impacts of continuous or pulsed exposures that better reflect human usage patterns throughout the mosquito season. Such studies would elucidate possible long-term population consequences for seasonal pulses in mosquito repellents and should be accompanied by analytical chemistry to quantify particular compounds comprising picaridin-containing repellents and their environmental concentrations and ecotoxicity. Additionally, future work should examine various sub-lethal effects of exposure, such as changes in behaviour and the carry-over fitness effects that would combine to influence ecosystem effects of exposure to this and other commercially available insect repellents.

Our findings also stress the importance of addressing poor sanitation and wastewater treatment infrastructure, which have already been shown to exacerbate the risk of mosquito-borne diseases in humans [22], and may indicate inefficient removal of picaridin from wastewater. Hence, improving sanitation in regions vulnerable to mosquito-borne diseases will both reduce transmission risk by reducing mosquito densities, and increase the removal of picaridin repellent, which may have unforeseen consequences for the ecological interactions that regulate mosquito population densities.

Given its efficiency in reducing transmission of mosquito-borne disease [3], and the lack of significant human toxicological risks with topical application [16], there are clear human benefits from the use of picaridin-containing repellents. However, these benefits may be offset by potentially heavy mortality of natural predators that may play an important role in the control of larval mosquito populations.

Ethics. The study protocol and animal care protocols were approved by the Institutional Animal Care and Use Committee at the Cary Institute of Ecosystem Studies (Proposal #03-15, #02-15).

Data accessibility. Raw data from the experiment evaluating salamander sensitivity to repellents containing DEET and picaridin can be accessed in the electronic supplementary material, S3.

Authors' contributions. R.M.A., B.A.H., E.J.R. and A.J.R. conceived the study design and conducted the experiments exposing larval salamanders to repellents containing DEET and picaridin. C.K. conducted the experiment on salamander predation of mosquito larvae. R.M.A. and A.J.R. carried out the statistical analyses. All authors contributed to the analysis and interpretation of the data. R.M.A. and B.A.H. drafted the paper, which was substantially edited and commented by all authors. All authors agree to be held accountable for the content of this manuscript and approve the final version of it.

Competing interests. We have no competing interests.

Funding. Funding for R.M.A. was provided by the Bentley Holden Fund Award and Brazil's Programa Ciências Sem Fronteiras – Doutorado Sanduíche (CNPq 201848/2015-5 - SWE). Funding for C.K. was provided by NSF REU grant no. 1062987.

Acknowledgements. Authors thank S. Ladeau, V. Ramesh, N. Jakubek, F. Roland and S. Bowden for helpful discussion and data collection assistance; L. Tumblety created figure 2.

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