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Mosquito repellents in frog skin

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The search for novel insect repellents has been driven by health concerns over established synthetic compounds such as diethyl-*m*-toluamide (DEET). Given the diversity of compounds known from frog skin and records of mosquito bite and ectoparasite infestation, the presence of mosquito repellents in frogs seemed plausible. We investigated frog skin secretions to confirm the existence of mosquito repellent properties. *Litoria caerulea* secretions were assessed for mosquito repellency by topical application on mice. The secretions provided protection against host-seeking *Culex annulirostris* mosquitoes. Olfactometer tests using aqueous washes of skin secretions from *L. caerulea* and four other frog species were conducted to determine whether volatile components were responsible for repellency. Volatiles from *Litoria rubella* and *Uperoleia mjobergi* secretions were repellent to *C. annulirostris*, albeit not as repellent as a DEET control. The demonstration of endogenous insect repellents in amphibians is novel, and demonstrates that many aspects of frog chemical ecology remain unexplored.

Keywords: Australia; frogs; *Litoria caerulea*; mosquito repellent; skin secretions; olfactometer

1. INTRODUCTION

Health concerns over synthetic mosquito repellents have stimulated research into natural alternatives (Fradin 1998). However, large scale studies of natural products have failed to find an alternative to the most widely used repellent, diethyl-*m*-toluamide (DEET). Frog skins are a pharmacopeia of bioactive compounds including peptides, guanidine derivatives, biogenic amines, steroids, alkaloids and volatiles (Daly *et al.* 1987; Bevins & Zasloff 1990; Erspamer 1994; Daly 1995, 1998; Toledo & Jared 1995; Smith 2001; Smith *et al.* 2004*a*,*b*). Given that mosquito bite, insect parasitism and predation on amphibians is widespread (Marks 1960; Van Beurden 1980; Kay *et al.* 1985; Ferrar 1987; Colless & McAlpine 1991), it is likely that there are compounds in the skin of some frog species to combat this (Williams *et al.* 1998). Using two types of laboratory experiment, we aimed to determine whether skin secretions from some Australian frog species are repellent to mosquitoes and whether this repellency is linked to volatile compounds in the secretion.

2. MATERIAL AND METHODS

Secretions were expressed from the skin of five Australian frog species using surface electrical stimulation (Tyler *et al.* 1992). This involves the application of low electric current to the skin, causing smooth muscle contraction and the expulsion of glandular contents. The secretions, which varied in odour (Smith *et al.* 2004*a*) and appearance (Williams *et al.* 2000) were washed from the frogs with 20 ml of distilled water and used immediately. Secretions were expressed from individual frogs for use in a particular test. Frogs were not reused. Although these methods provided skin secretions that varied in concentration, our aim was merely to establish the existence of mosquito repellent properties in frogs, not to quantitatively compare repellency.

Frogs were collected from the following locations in Australia: *Litoria caerulea* and *Litoria rothi*, Nutwood Downs, NT; *Litoria splendida*, Lake Argyle, WA; *Litoria rubella*, Farina, SA; *Uperoleia mjobergi*, Derby, WA. The choice of species was governed by collection opportunities. Frogs varied in the duration of captivity prior to use: *L. caerulea ca* two months, *L. rothi ca* 2 years, *L. splendida ca* 2 years, *L. rubella ca* six months, *U. mjobergi ca* 2 years.

To determine whether frog skin secretions could repel biting mosquitoes, the secretion from *L. caerulea* was tested in six 1 h trials to determine the protection it offered mice against mosquito bite. A colony of *Culex annulirostris* was maintained at 25 °C and 70% relative humidity in a 12 : 12 h L : D regime. This species has a wide host range (including amphibians) and is widely distributed throughout Australia (Kay *et al.* 1985). Blood meals were provided by restrained mice and larvae were reared in boiled pond water on a combination of dried fish and dog foods.

Biting time tests were conducted in a $70\times70\times70$ cm cage. Mice (Swiss strain) were restrained in small acrylic cylinders with an anterior mesh screen and a posterior cork with a slot allowing the tail to protrude. The tail was fastened loosely to the cylinder with tape. Pairs of mice (with only the tail accessible to mosquitoes) were exposed to 80 host-seeking female *C. annulirostris* mosquitoes in a cage. One mouse had its tail treated with 1 ml of a *L. caerulea* secretion–distilled water solution (approx. 5–10%), whereas the other received a distilled water control. Both extracts were applied to the entire tail with a cotton applicator. The Mann–Whitney *U*-statistic (Zar 1999) was used to test for a significant difference in the time to first bite for treated and control mice.

Skin secretions from all five species and a 0.1% DEET solution in ethanol were tested against *C. annulirostris* mosquitoes using a vertical olfactometer, with a blank control air-stream used each time. The olfactometer consisted of a small chamber atop a laminar air flow cabinet (figure 1). The chamber was large enough for 10 mosquitoes to fly and be exposed to two $26\ \mathrm{cm\ s^{-1}}$ air streams: a blank control and one containing volatiles from frog skin secretion. Before each experiment, the test chamber was cleaned using 70% ethanol followed by distilled water. It was then allowed to air dry, with the filtered air of the olfactometer allowed to blow through the chamber for 1 h prior to experiments. Ten female mosquitoes acclimatized to the test chamber 10 min prior to each trial with the fan running. Four cylinders of Whatman No. 1 filter paper (3 cm diameter, 5 cm height) were placed on the brass frame in both sides of the test chamber. One set of cylinders received the frog skin secretion, the other a distilled water control. Cylinders were dipped for 5 s in either the secretion or water, allowing the liquid to migrate to halfway up the cylinders by capillary action. This was a sufficient volume of secretion to give a detectable odour. Air passed through the cylinders and a mesh screen, carrying volatiles in the frog secretion into the test chamber containing mosquitoes.

Mosquitoes were monitored for 8 min following introduction of the test substance and control. At the end of each minute, the number of mosquitoes on the treatment and control sides of the chamber was recorded. The mean number on each side of the chamber throughout each trial was calculated and used for analysis. Four trials with different groups of 10 mosquitoes were used to test against the secretion of each frog species. Heterogeneity chi-square tests (Zar 1999) were used to determine whether the four trials for each species could be pooled. In all cases, the null hypothesis of heterogeneity was rejected, so data from the trials were pooled to calculate mean values for the number resting in control and treatment air streams for each species. This homogeneity indicated the absence of significant variability in the mosquito repellency of individual frogs within a species. These values were then tested in a goodness-of-fit chi-square test (with Yates correction) to test deviation from a 50 : 50 ratio on the control and treatment

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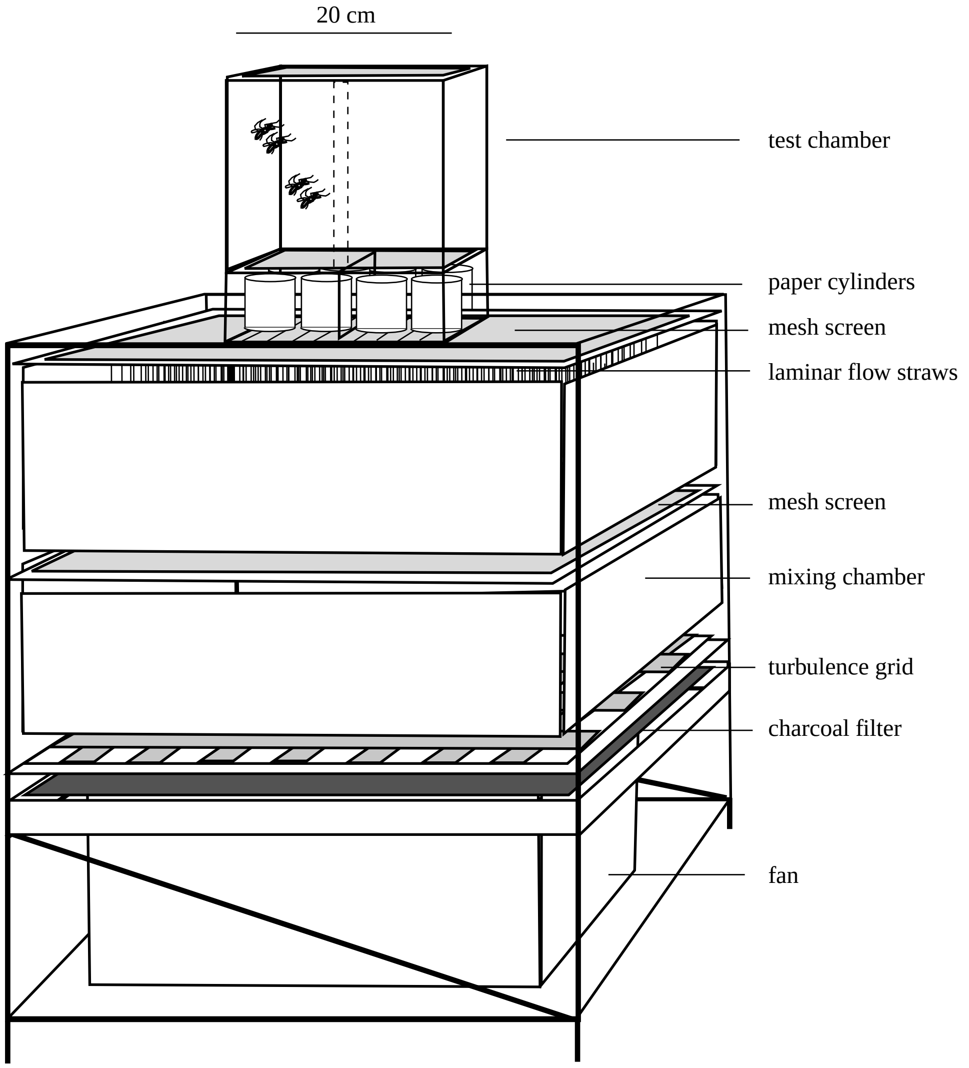

Figure 1. Schematic drawing of the vertical olfactometer. The lower section produces filtered, homogenized, laminar flow air at 26 cm s^{-1}. The upper section provides two air streams into which frog skin secretion volatiles can be introduced.

air streams (Zar 1999). A repellency quotient (RQ) was calculated for each species using the following formula: $RQ = (N_{\text{repelled}} - N_{\text{unrepelled}})/(N_{\text{repelled}} + N_{\text{unrepelled}})$. The RQ returns a value of 1 for complete repellency and 0 for no effect.

3. RESULTS

Litoria caerulea secretion provided protection from biting for up to 50 min (mean time to first bite: 46±2.31 min), which was significantly greater than the control (12±1.47 min) ($U<0.001$, $p=0.029$). A 1 ml application of 10% DEET solution in ethanol offered protection for up to 2 h.

Secretions from *L. rubella* and *U. mjobergi* were found to be repellent in the olfactometer, whereas *L. caerulea* secretion was marginally repellent (table 1). DEET was almost twice as repellent as *L. rubella*, which had the most repellent secretions. No repellency was found for *L. rothi* and *L. splendida*.

4. DISCUSSION

The mosquito biting experiment using *L. caerulea* secretion demonstrated that at least one frog species expresses mosquito repellent chemicals in its skin. The olfactometer experiment demonstrated that mosquito repellency is also a property of the secretions of two other species and is probably attributable to a volatile component. Mosquito repellency is not restricted to one species or genus of frog.

However, despite offering protection against mosquito bite, the repellency effect for *L. caerulea* secretion in the olfactometer was marginal. This is most likely due to the use of aqueous washes (a limitation of the olfactometer study) and variation in the individual potency of frog skin secretions. Due to the 'sticky' proteinaceous matrix of the skin secretion, the efficacy of aqueous washes to remove the repellent compounds may have been limited. It seems probable that the secretion matrix plays a role in retaining and/or releasing the repellent compounds from the skin surface. This is evidenced by unpublished sensory studies, which showed that aqueous washes lost their odour much more rapidly than cotton swabs of the secretion (B. P. C. Smith 2001, unpublished work). In addition, the odour potency of individual frogs varied considerably. Consequently, the actual concentration of repellent compounds in the washes may have varied considerably. Thus, only the presence or absence of mosquito repellency can be established for a frog species from this study, and no quantitative comparisons are possible. Typically, the odours

Table 1. Mosquito repellency for the secretions of five frog species against *C. annulirostris* mosquitoes in an olfactometer. (A repellency quotient (RQ) value of 0 indicates no repellency, a value of 1 indicates complete repellency.)

species	trial no.	mean repelled	mean not repelled	RQ	χ^2	p
L. caerulea	1	7.50	2.50	0.275	2.5	0.114
	2	6.50	3.50			
	3	5.00	5.00			
	4	6.50	3.50			
L. rothi	1	5.75	4.25	0.016	0.003	0.956
	2	5.63	4.37			
	3	4.44	5.56			
	4	4.50	5.50			
L. rubella	1	7.75	2.25	0.450	7.225	0.007
	2	8.63	1.37			
	3	5.88	4.13			
	4	6.75	3.25			
L. splendida	1	7.00	3.00	0.113	0.306	0.580
	2	5.13	4.87			
	3	4.13	5.87			
	4	6.00	4.00			
U. mjobergi	1	7.25	2.75	0.369	4.727	0.030
	2	5.13	4.87			
	3	8.75	1.25			
	4	6.25	3.75			
0.1% DEET	1	9.00	1.00	0.800	17.6	<0.001
	2	9.00	1.00			
	3	9.00	1.00			
	4	9.00	1.00			

accompanying the expulsion of glandular contents are ephemeral to the human nose, indicating that a pulse of volatile chemicals is released by the frog.

Given the diversity of compounds already identified from frog skin secretions, the discovery of insect repellent properties is not wholly surprising. The specificity of repellency still needs to be determined, but it is likely to be broad acting. Many synthesized repellents such as DEET, and naturally occurring ones such as azadirachtin (neem tree extract) have repellent and/or anti-feedant action against a diversity of insect types, including mosquitoes (Ravindran *et al.* 2002; Frances *et al.* 2004; Hou *et al.* 2004). The demonstration of blowfly anti-feedant properties in *L. caerulea* skin secretion (Williams *et al.* 1998), which is also active against mosquitoes, supports such an hypothesis. It follows that the evolution of mosquito repellents in frog skin may not have always been towards mosquitoes, but rather other parasitizing or predating insects.

The absence of repellent properties in *L. rothi* and *L. splendida* secretions is possibly because specimens tested here were deficient in repellent compounds due to their time in captivity (*ca* 2 years); although secretions from *U. mjobergi* held captive for a similar duration were highly repellent. Factors such as diet (not as varied as a wild diet) and housing conditions in captivity have been shown to influence frog skin secretions (Smith 2001; Smith *et al.* 2004*b*). Terpenes (known repellent compounds) can be sequestered from dietary sources and expressed in skin secretions by at least two frog species, *L. caerulea* and *L. ewingi* (Smith 2001; Smith *et al.* 2003, 2004*b*).

It is possible that terpenes identified by Smith *et al.* (2003, 2004*b*) are responsible for some of the repellency observed here. Although not water soluble, terpenes may persist in small amounts in aqueous washes, bound in a matrix of the proteins present in these secretions. Although the chemical characterization of frog skin volatiles compounds has already begun (Smith *et al.* 2003, 2004*b*), further work, perhaps focusing on *L. rubella* and *U. mjobergi*, may reveal further naturally derived mosquito repellents.

Although endogenous insect repellents have been reported in the crested auklet, *Aethia cristatella* (Douglas *et al.* 2001), such reports are rare. This report of mosquito repellent compounds in an amphibian is novel, and demonstrates that many aspects of frog chemical ecology remain unexplored.

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