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SHORT COMMUNICATION

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Benzoquinones from millipedes deter mosquitoes and elicit self-anointing in capuchin monkeys (*Cebus* spp.)

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Abstract Neotropical monkeys of the genus *Cebus* anoint themselves by rubbing arthropods and plants against their pelage. A recent study has shown that free-ranging wedge-capped capuchin monkeys (*C. olivaceus*) in Venezuela self-anoint with a benzoquinone-secreting millipede, an activity by which they are hypothesized to appropriate chemical deterrents of mosquitoes. To evaluate the plausibility of this hypothesis, female yellow fever mosquitoes (*Aedes aegypti*) were presented with two millipede secretory compounds, 2-methyl-1,4-benzoquinone and 2-methoxy-3-methyl-1,4-benzoquinone, on nylon-reinforced silicone membranes placed over wells filled with human blood, a highly preferred food. Mosquitoes exhibited fewer landings, fed less frequently, and flew more frequently (a possible indication of repellency) in the presence of membranes treated with benzoquinones than with controls. These compounds also elicit self-anointing in captive male and female tufted (*C. apella*) and white-faced (*C. capucinus*) capuchin monkeys.

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Introduction

Many mammals and birds topically apply leaves, arthropods, or other scent-bearing materials to their integument. Some investigators suggest that these anointing behaviors serve to appropriate chemical deterrents of ectoparasites (Simmons 1966; Clayton and Wolfe 1993). A recent study has shown that free-ranging wedge-capped capuchin monkeys (*Cebus olivaceus*) in Venezuela rub themselves with the millipede *Orthoporus dorsovittatus* (order Spirostreptida) (Valderrama et al. 2000). This anointing behavior was hypothesized to function in appropriating the defensive secretions of this myriapod's numerous segmental glands as a means of repelling mosquitoes.

We report here the results of in vitro experiments demonstrating that the two known secretory components of *O. dorsovittatus*, 2-methyl-1,4-benzoquinone (toluquinone) and 2-methoxy-3-methyl-1,4-benzoquinone (MMB), deter biting mosquitoes. These benzoquinones, which are the chief defensive compounds of the millipede orders Julida, Spirobolida, and Spirostreptida (Eisner et al. 1978), occur in an approximately 1:1 ratio in the glandular exudate of *O. dorsovittatus* (Valderrama et al. 2000). We also report that these chemicals elicit self-anointing in the tufted capuchin monkey (*C. apella*), which ranges throughout northern and central South America, and the white-faced capuchin monkey (*C. capucinus*), which ranges from Belize and Honduras to western Colombia and Ecuador. Both species of *Cebus* have been reported to self-anoint with arthropods and plants (Nolte 1958; Baker 1996).

Few ectoparasitic arthropods that attack vertebrates have been tested for responses to anointing materials (Clayton and Vernon 1993; Clayton and Wolfe 1993). We tested the yellow fever mosquito (*Aedes aegypti*), which occurs in humid habitats worldwide between 45°N and 35°S (Christophers 1960), for responses to millipede-produced benzoquinones. Females of this species feed on a variety of vertebrates, especially mammals and birds, and are vectors of yellow fever and dengue viruses, filariasis, and other diseases.

Materials and methods

Subjects

Laboratory colonies of *A. aegypti* were reared at 28°C and 80% RH, and kept on a 12:12 (L:D) photoperiod. Adult female mosquitoes, 5–16 days old, were maintained on a 10% sucrose solution presented on cotton pads; 48 h before testing, the sucrose-soaked pads were replaced by pads soaked in water, and 24 h beforehand, the water was removed.

Eight male and two female *C. apella* and six male and six female *C. capucinus* (2.3–5.4 kg, $\bar{X}$=3.4 kg) were housed according to species at Jungle Friends Primate Sanctuary, Gainesville, Fla., in wire-mesh enclosures (ranging from 12.2×5.5×4.6 m to 1.8×1.8×1.8 m) in groups of 1–6 individuals. Most of the animals were captive-born and ranged in age from 18 months to 40 years old; the histories of two individuals are unknown.

Chemicals

Toluquinone was obtained commercially (Sigma-Aldrich, St. Louis, Mo.). MMB was synthesized from 2′,4′-dihydroxy-3′-methylacetophenone (Sigma-Aldrich) via 4′-benzyl-2′-methoxy-3′-methylacetophenone (Raphael and Ravenscroft 1998). Baeyer-Villiger oxidation with *m*-chloroperoxybenzoic acid in the presence of toluenesulfonic acid (Knölker and Fröhner 1997) gave the acetate of 4-benzyloxy-2-methoxy-3-methylphenol. Saponification (sodium hydroxide in methanol) and hydrogenation (5% Pd/C, ethanol, 1 atm) yielded 2-methoxy-3-methyl-1,4-hydroquinone, which was oxidized to MMB with ferric chloride (Luly and Rapoport 1981).

Tests of mosquitoes

Experiments were conducted from 0900 hours to 1500 hours in a walk-in incubator (26°C, 63–80% RH) illuminated by seven 32 W fluorescent light bulbs. Mosquitoes were tested in a Plexiglass module designed to assess their responses to chemicals applied to nylon-reinforced silicone membranes (Butler et al. 1984) laid over shallow wells filled with human blood, a highly preferred food of female *A. aegypti* (Harrington et al. 2001). The responses of mosquitoes to serial dilutions of toluquinone, MMB, and a 1:1 mixture of these compounds were examined to estimate threshold sensitivities with our blood-meal presentations. Each compound and the 1:1 mixture were presented as 0.025, 0.05, 0.1, 0.2, and 0.4 M solutions in acetone. Acetone was applied to the membranes as a control in all experiments. Twelve trials, each using five mosquitoes, were conducted for each compound and the 1:1 mixture at each dilution.

The module consisted of two interfacing pieces. The bottom piece was a 40×7×4 cm hollow platform with water inlet and outlet spouts. It supported six circular wells (diameter = 3.8 cm, depth = 6 mm) spaced 2.5 cm apart. Water (40°C) flowed through the central cavity of the platform at 215 ml/min. The top piece consisted of six 4.5×4.0×5.0 cm chambers, separated from one another by a 1.6 cm-wide enclosed space. The floor of each chamber was fitted with a sliding door positioned over a circular opening 3.5 cm in diameter. An aperture 1 cm in diameter on the front wall of each chamber served as a portal through which mosquitoes were introduced.

For each test, five female mosquitoes were aspirated into each chamber of the module, and the apertures through which they were introduced were plugged with wooden corks wrapped in plastic film. The wells on the bottom piece were filled with 7 ml of warmed (40°C) outdated human blood (Walter Reed Army Medical Center Blood Bank, Washington, D.C.) to which 2.9 mg/ml of ATP had been added. Five minutes prior to testing, 50 µl of the benzoquinone solutions or acetone were applied to 9.6 cm^2 circular areas on 0.1 mm-thick membranes made of nylon mesh in a silicone matrix (Butler et al. 1984). The treated areas of the membranes were then placed over each well in contact with the blood.

The top piece of the module was placed over the bottom piece by aligning the circular openings on the floor of each chamber with each membrane-covered well. The sliding floors of the chambers were then opened, allowing mosquitoes access to the membranes. The number of mosquitoes landing on experimental and control membranes, and those flying within the apparatus, were recorded at the end of each minute for 5 min. Flying was scored because it is elicited in *A. aegypti* by some repellents (Daykin et al. 1965); our apparatus, however, was not strictly designed to measure repellency, which entails orientation away from a chemical source (Dethier et al. 1960).

After 5 min, any mosquitoes remaining on the membranes were prodded off with a metal wire, and the door on the floor of each chamber was closed to confine them in the chamber. The top piece of the module was then placed in a freezer for 20 min. To determine whether mosquitoes had fed through the membranes, they were removed from the frozen module with forceps, crushed on white paper towels, and examined for the presence of blood.

The proportions of mosquitoes feeding per trial were analyzed using a standard generalized linear model with a logit link. Landing and flying scores are repeated measures; five readings were obtained for groups of five mosquitoes. Thus, to maintain independence among our data, we summed the five readings, then divided each sum by 25 (maximum score in both categories) to create proportions of mosquitoes landing and flying. The proportions were then transformed using the standard variance stabilizing transformation for proportions ($\sin^{-1}\sqrt{y}$, where y is the proportion), and analyzed using ANOVA.

Tests of monkeys

Monkeys were individually isolated in their home enclosures and presented with filter papers (diameter = 4.5 cm) treated with 50 µl of 0.1 M solutions of toluquinone or MMB in acetone, or acetone alone. Tests were conducted from 0730 hours to 1130 hours. Each individual was offered, by hand, filter papers, and was then observed until it dropped, shredded (usually by biting) or wiped the papers against itself. The order in which filter papers was presented was alternated with each subject. There was an interval of 1 min between trials.

Results

In tests of *A. aegypti* with toluquinone (Fig. 1a), contrasts with the control vs 0.2, and 0.4 M solutions for feeding were significant at $P<0.0001$ (χ^2 test, df=66, χ^2=25.64 and 26.63, respectively). Contrasts with the control vs 0.1, 0.2, and 0.4 M solutions for landing were significant at $P<0.01$ (t test, df=66, t=3.05, 6.01, and 6.36, respectively). Contrasts with the control vs 0.1, 0.2, and 0.4 M solutions for flying were significant at $P<0.0001$ (t test, df=66, t=−2.35, −3.38, and −5.52, respectively). In the experiment with MMB (Fig. 1b), contrasts with the control vs 0.025, 0.05, 0.1, 0.2, and 0.4 M solutions for feeding were significant at $P<0.02$ (χ^2 test, df=66, χ^2=6.23, 14.53, 30.37, 42.07, and 25.79, respectively). Contrasts with the control vs 0.025, 0.05, 0.1, 0.2, and 0.4 M solutions for landing were significant at $P\leq0.0003$ (t test, df=66, t=2.67, 3.70, 5.02, 5.14, and 3.80, respectively). Contrasts with the control vs 0.1, 0.2, and 0.4 M solutions for flying were significant at $P<0.005$ (t test, df=66, t =−2.98, −7.34, and −3.90, respectively). In the experiment with the 1:1 benzoquinone mixture (Fig. 1c), contrasts with the control

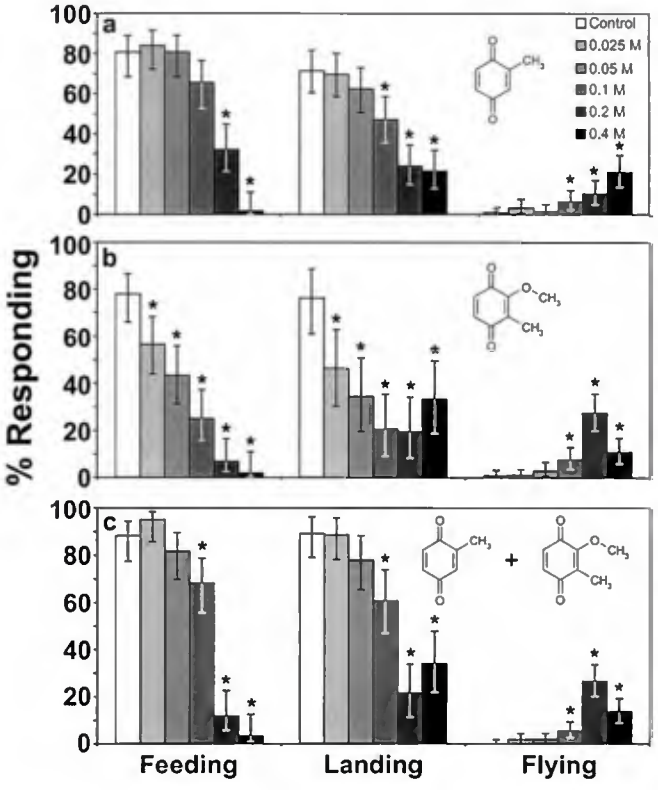

Fig. 1 Mean percentages and 95% confidence intervals of *Aedes aegypti* feeding, landing, and flying in response to silicone membranes covering blood-filled wells and treated with acetone and 0.25, 0.05, 0.1, 0.2, and 0.4 M acetone solutions of toluquinone (**a**), MMB (**b**), and a 1:1 mixture of toluquinone and MMB (**c**). Sixty mosquitoes were tested with each dilution of each solution. An *asterisk* (*) indicates values significantly different from control. Values for landing and flying percentages were backtransformed; the analysis was done on the arcsine transform scale, $y=\sin^{-1}\sqrt{p}$, where y is the transformed score and p is the original proportion

vs 01, 0.2, and 0.4 M solutions for feeding were significant at $P<0.02$ (χ^2 test, $df=66$, $\chi^2=6.60$, 50.68, and 42.82, respectively). Contrasts with the control vs 0.1, 0.2, and 0.4 for landing were significant at $P\leq 0.0009$ (t test, $df=66$, $t=3.47$, 7.66, and 6.20, respectively). Contrasts with the control vs 0.1, 0.2, and 0.4 M solutions for flying were significant at $P<0.002$ (t test, $df=66$, $t=-3.40$, -9.11, and -6.07, respectively).

The adverse effect of benzoquinones was also evidenced by mosquitoes that became inverted or died on the membranes when exposed to higher concentrations of these compounds. Eight (13%) mosquitoes exposed to 0.4 M toluquinone, 22 (37%) of those exposed to 0.4 M MMB, and three (5%) and 16 (27%) of those exposed to 0.2 M and 0.4 M solutions of the 1:1 benzoquinone mixture, respectively, failed to fly off the membranes at the end of the 5 min tests, so they were removed with forceps and crushed to examine for blood. One immobile mosquito was removed from a control membrane.

Benzoquinones elicited energetic anointing in monkeys, whereby they wiped the treated filter papers against themselves; rubbed their fur with hands, feet, and tail; and

Fig. 2 A *Cebus apella* rubs a filter paper treated with toluquinone against its head (*top*), then, reaching back, against the base of its tail

occasionally drooled (Fig. 2). Five (50%) and three (30%) *C. apella* and eight (67%) and four (33%) *C. capucinus* anointed with papers treated toluquinone and MMB, respectively. Four male and one female *C. apella* and six male and two female *C. capucinus* anointed with one or both compounds. Anointing bouts began within 10 s after grasping filter papers and lasted up to 66 s ($\overline{X}=25$ s).

Self-anointing was not observed with control filter papers, which were released within a few seconds.

Discussion

Our in vitro experiments demonstrate that toluquinone and MMB, the chief glandular products of numerous millipedes, deter contact and feeding by *A. aegypti*. MMB

reduced landing and feeding at lower concentrations than did toluquinone. Toluquinone also inhibits feeding in the pharaoh ant (*Monomorium pharaonis*) (Peschke and Eisner 1987) and flour beetles (*Tribolium* spp.) (Loconti and Roth 1953; Ogden 1969; Mondal and Port 1984), and acts as a contact irritant of *Tribolium* spp. (Ogden 1969) and the American cockroach (*Periplaneta americana*) (Peschke and Eisner 1987). The toxicity of toluquinone and other benzoquinones has also been demonstrated with several insects (Kanehisa 1969), thus corroborating our observations of impaired or dead mosquitoes on feeding membranes exposed to the more concentrated solutions. The trend of increased landings and decreased flying to the 0.4 M solutions of MMB and the 1:1 benzoquinone mixture, compared to the corresponding 0.2 M solutions (Fig. 1b, c), likely is due to intoxicated subjects collapsing on the membranes.

Our demonstrations that benzoquinones deter biting mosquitoes and elicit anointing behaviors in *Cebus* spp. support the suggestion that the topical application of these compounds constitutes a defense against some ectoparasitic arthropods (Valderrama et al. 2000). The effective deterrence of mosquitoes in nature, however, depends upon a variety of factors that remain to be assessed, including the ability of monkeys to obtain sufficient quantities of deterrent compounds from the millipedes they encounter. Estimates of the amounts of benzoquinones produced by some tropical millipedes indicate up to 350 mg per individual (Fairhurst 1993), with quinones comprising more than 1% of the body weight of some species (Eisner et al. 1978). Monkeys may enhance the quantities of secretions they obtain by biting and crushing millipedes to access the internal reservoirs of glandular fluids (Valderrama et al. 2000).

Apart from *C. olivaceus*, other Central and South American monkeys (Baker 1996; Zito et al. 2003), Malagasy lemurs (Overdorff 1993; Birkinshaw 1999), and a variety of birds in the Old and New Worlds (reviewed by Parkes et al. 2003) self-anoint with millipedes. Most millipedes identified as those used for these activities are members of the Julida, Spirobolida, or Spirostreptida, which characteristically secrete benzoquinones. Studies are needed on the possible elicitation of self-anointing by these compounds in other vertebrates.

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