Does toxic defence in *Nycticebus spp.* relate to ectoparasites? The lethal effects of slow loris venom on arthropods

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ABSTRACT

The venom produced by slow lorises seems to be toxic both intra- and inter-specifically. In this study we assessed the adaptive properties of their venom to repel ectoparasites. We tested venom from two Indonesian slow loris species: *Nycticebus javanicus* and *N. coucang*. Arthropods directly exposed to brachial gland secretions mixed with saliva from both slow loris species were immediately impaired or exhibited reduced activity (76%), and often died as a result (61%). We found no significant difference in the result of 60-minute trials between *N. coucang* and *N. javanicus* [X^2 (2, n=140)=2.110, p=0.3482]. While most maggots (84%) were initially impaired from the venom after 10 minutes, maggots died after a one-hour trial 42% of the time. In contrast, at the end of one hour arachnids died 78% of the time. For all arthropods, the average time to death from exposure was less than 25 minutes (M=24.40, SD=22.60). Ectoparasites including ticks, members of the arachnid order, are known to transmit pathogens to hosts and may be an intended target of the toxic secretions. Our results suggest that one function of slow loris venom is to repel parasites that affect their fitness, and that their topical anointing behaviour may be an adaptive response to ectoparasites.

INTRODUCTION

Few mammals are known to produce toxic secretions (Ligabue-Braun et al. 2012). The functions of mammal venom vary but include suppressing prey, anti-predator defence and intraspecific competition (Ligabue-Braun et al. 2012). Slow lorises (*Nycticebus spp*.) are the only primates known to produce venom and do this by combining saliva with oil from a brachial gland in their mouth (Alterman 1995), and licking their fur or biting the intended victim. Here we explore the adaptive significance of venom amongst Indonesian slow lorises in regard to its effects on invertebrates.

Nekaris et al. (2013) suggested that slow loris venom might function to repel or defend against predators, conspecifics, prey or ectoparasites. In terms of the latter hypothesis, chemical toxicity is one feature that renders vertebrates as unsuitable hosts for ectoparasites (Weldon 2010). Slow lorises have rarely been observed to harbour ectoparasites (Streicher 2004; Nekaris, et al., 2013), and it has been proposed that this is due to the chemicals produced by their saliva, brachial grand secretions, or a combination of the two. Ectoparasites are important selective forces that negatively affect the fitness of their hosts (Moller et al. 1993; Weldon and Carroll 2006), and are common in the tropical Southeast Asian countries that slow lorises inhabit (Anastos 1950).

We explored whether the secretions produced by slow lorises are lethal to ectoparasites. We examined the behavioural and physiological responses of arthropods to slow loris venom. We predicted that: a) arthropods will die more rapidly after direct exposure to slow loris secretions and b) arthropods will avoid moving to test areas that have been applied with slow loris secretions.

MATERIALS AND METHODS

Study Site

We tested the repellent effects of venom produced by adult wild Javan slow lorises (*N. javanicus*) in an agroforest study site in Garut District, West Java, Indonesia (S7°6′6 & E 107°46′5) and adult wild-born greater slow lorises (*N. coucang*), recently confiscated from the illegal wildlife trade in Sumatra, at Cikananga Wildlife Centre, Sukabumi District, West Java (S7°00′23.9 & E 108°33′3.9).

Between July 2013 and January 2014, we collected brachial gland and saliva samples from Javan slow lorises using Sterilin swab kits (n=49). In March 2014, we collected saliva and brachial gland samples from greater slow lorises using cotton swabs stored in sterile glass vials and Salimetrics oral swabs that we centrifuged (n=42). We froze all samples until usage.

Following Alterman (1995), we extracted frozen brachial oil with a 2 ml solvent of 6% formic acid, which solvates hydrophilic compounds, or alternatively with a 2 ml solvent of 1:1 50% methanol and 50% methylene chloride, which is lipid soluble. We only used the methanol:methylene chloride solvent with maggots, as the solvent alone impaired other arthropods. After incubating at room temperature for 30 minutes, we mixed 100 µl aliquots of saliva with the extracted solutions and incubated for an additional 15 minutes. Alternatively, we mixed saliva on swabs with the venom solution and incubated for 15 minutes. In experiments using only saliva, we applied saliva directly with no solvent.

Due to remote field conditions, we collected multiple types of insects for use in the experiments, including spiders (Arachnida), maggots (Diptera larvae), ants (Hymenoptera), fleas (Siphonaptera), and caterpillars (Lepidoptera larvae) controlled for length: 2 cm for maggots; 1-

1.5 cm for spiders, and 2 cm for caterpillars. We attempted to sample tick abundance in the study area (Carroll and Schmitmann 1992, Sonenshine 2004); we found no adult ticks in two weeks of surveys, so we substituted spiders as an analogy.

Experiments included three types of secretions: brachial gland secretion, saliva, and glandular secretions mixed with saliva. We topically applied the venom solution to subjects in a petri dish. We established controls with an insect of the same size and species treating it only with the solvent, or water in tests using saliva only. The following amounts were applied to the abdomen of each arthropod, avoiding the head: 100 µl for those less than 1 cm in length; 200 µl for 1.5 cm length; 300 µl for 2 cm length.

We conducted all tests on forward locomoting individuals. We recorded responses at 0-, 10-, 30-, and 60-minute intervals: no effect (subject continues locomoting with no response), reduced activity (locomotor activity slows or is disrupted), impaired (motor impairment; forward locomotor activity stops or individual appears to be struggling), death, and time until death.

RESULTS

We tested 140 subjects (Arachnida: n=57; Siphonaptera: n=10; Diptera: n=41; Hymenoptera: n=12; Lepidoptera: n=10) in 93 experimental trials with 30 control trials. The average time to death for trials that resulted in death (n=50) was 24.40 minutes (SD=22.60).

Tests using saliva or brachial oil only on fleas (n=3), maggots (n=3), and spiders (n=10) did not have a significantly different outcome than using water as the control (Pearson Chi Square: X^2=1.272, *p*-value=0.529, α=0.050). These tests were stopped to preserve the limited samples, and a combination of brachial oil and saliva was used for the remaining tests (n=73). Due to the limited amount of saliva available, we stopped direct tests of saliva after only five

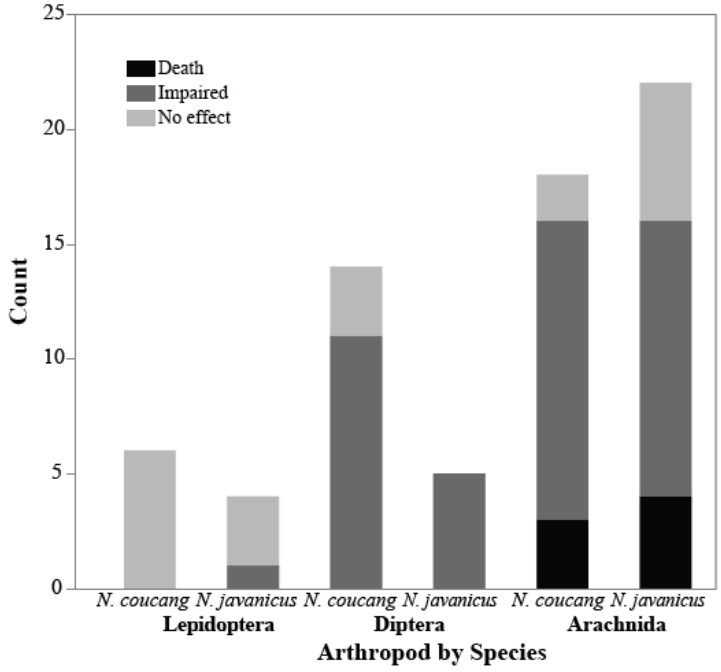
93 trials in order to preserve the amount of samples. Similarly, 11 tests using brachial secretions
94 only sometimes resulted in initial impairment and more often had no effect for fleas (n=3),
95 maggots (n=3), and spiders (n=5). We used a combination of brachial oil and saliva for the
96 remaining direct application tests (n=73).
97 Arthropods directly exposed to slow loris brachial gland secretions mixed with saliva
98 from both species were immediately impaired (76% of time), and often died as a result (61% of
99 time); treated arthropods had a significantly different response than those exposed to the solvent
100 alone (Pearson Chi Square: X^2=23.38, *p*-value <0.0001, α=0.050). The immediate results of the
101 application are not included in the analysis, as there are confounding factors from the
102 physiological responses to having a foreign substance applied. We found no significant
103 difference in time of death between spiders and maggots (Student's *t*-*t*=2.048, p=0.315, α=0.05).
104 While most maggots (84%) were initially impaired from the venom after 10 minutes, maggots
105 died after a one-hour trial only 42% of the time (Table 1). In contrast, at the end of one-hour
106 arachnids died 78% of the time (Table 1). For spiders, after only ten minutes direct application
107 of a venom treatment, 80.00% were dead, impaired or exhibiting reduced activity (Table 1).
108 After one hour, 65.00% of spiders were dead, and 38.00% remained impaired and often died
109 immediately after.
110 We found no overall significant difference in the result of the 60-minute trials between *N.*
111 *coucang* and *N. javanicus* (Pearson X^2: *X*=2.110, df=1, p-value=0.35, α=0.05; Table 1). For
112 spiders, time until death between *N. javanicus* and *N. coucang* did differ significantly (Kruskal
113 Wallis: Z=-1.974, p-value=0.05, α=0.05), where subjects applied with venom of *N. javanicus*
114 took a mean of 39.18 minutes to die compared to 23.38 minutes for *N. coucang* (Fig. 1). We
115 found no significant difference between the effects of male and female venom in time to death

(Pearson Chi Square: X^2=7.454, p-value=0.11, α=0.05) or in time to death for male, female, and combination male/female venom (ANOVA: F=3.0613, *p*=0.0563, α=0.05).

Table 1. Percentage of arthropods that exhibit no effect, impairment, or death at the end of 10- and 60- minute single experimental trials.

	RESULT OF TRIALS					
Type	Death		Impaired		No Effect	
	10 min	**60 min**	**10 min**	**60 min**	**10 min**	**60 min**
Ant **(n=10)**	0.90	0.90	0	0	0.10	0.10
Caterpillar **(n=10)**	0.00	0.00	0.10	0.00	0.90	0.10
Maggot **(n=19)**	0.00	0.42	0.84	0.26	0.16	0.32
Spider **(n=40)**	0.18	0.78	0.63	0.08	0.20	0.15

Figure 1. Bar chart indicating the proportion of results of the one-hour trial for all tested arthropods (full lethality, motor impairment, or no effect) according to loris species.



DISCUSSION

We show here that the venom of slow lorises is toxic and often lethal to a variety of insect species. Consistent with previous findings (Alterman 1995), we confirm that this is only the case when brachial grand secretion is combined with saliva. The degree of lethalness of the venom is taxon-specific and varies according to the type of arthropod to which it is exposed. The venom was more lethal for arachnids and ants than for maggots and caterpillars, suggesting its use as a deterrent against some ectoparasites.

We suggest in particular that that the anointing behaviour observed in slow lorises may be related to repelling ectoparasites. Slow lorises spend up to 10% of their active time autogrooming (Rode et al., 2014). Being extremely flexible, slow lorises can lick most parts of the body with the tongue, but also profusely lick their arms and rub them on their head, face and other obtainable body parts (Schulze et al., 1995). The brachial gland secretions of slow lorises, when combined with their saliva and manually or orally applied to their fur, would be a feasible means of reducing ectoparasite load.

Other vertebrates are known to use toxic compounds with pesticide qualities. Numerous bird species engage in anting behaviour, taking advantage of the repellent chemical properties that ants leave behind (Weldon & Carroll 2006). New Guinean pitohuis absorb chemicals from their melyrid beetle prey as a potential predator and parasite defense system (Dumbacher, et al. 2004). Other primates, including *Cebus*, *Aotus*, and *Ateles,* anoint themselves with insects and plants in order to ward off ectoparasites, especially ticks (Laska et al. 2007). Careful tri-monthly in-hand examination of all slow loris individuals from this study did not reveal any detectable ectoparasite loads, despite domestic animals in the agroforest environment being heavily infected (Albers et al., 2013).

154 The fact that slow loris venom has a more pronounced toxic effect on arachnids than
155 other types of arthropods is notable, as ticks are arachnids and a likely recipient of anointed slow
156 loris venom. It is unlikely that topically applied secretions would be repellent to other types of
157 ectoparasites, such as mosquitos, who leave hosts swiftly and do not spend a protracted period of
158 time on hosts as ticks do (Weldon et al. 2011). In ectoparasites, the venom may be sensed by
159 olfactory means and an avoidance response may be triggered. While ectoparasites themselves
160 may not represent a significant impact on the fitness of a host animal in and of themselves,
161 parasites - especially ticks - may carry pathogens that serve as a more potent threat.
162 Ticks are a likely threat for slow lorises, including both Javan and greater slow lorises.
163 Sumatra is home to 22 known species of ticks, while Java is home to 18 different species
164 (Anastos 1950). Although ticks are usually exclusively found at terrestrial and understory levels,
165 ticks have been sampled in the forest canopy, possibly transported from arboreal primate hosts
166 (Loaiza et al. 2013). Specifically, *N. coucang* is reportedly a known host for *Haemaphysalis*
167 *koningsbergeri* (Anastos 1950). The lorises in this study would have been more susceptible to
168 attracting ticks. The wild Javan slow lorises move frequently on the ground due to high levels of
169 disturbance (Rode et al. 2014). The greater slow lorises were kept in captivity in close proximity
170 to many other vertebrate species, but also during their time in trade would have been more
171 susceptible to high stress and low immunity (Streicher 2004). Lack of ectoparasites on both
172 populations provides further support that another factor may contribute to low ectoparasite load –
173 in this case, slow loris venom.
174 Ticks are commonly recognized carriers of pathogens for humans and animals (Dautel
175 1999, Sonenshine 1993). Tick-borne pathogens are common in Southeast Asia, although there is
176 a lack of knowledge on the extent, distribution, and prevalence of these pathogens on both

177 humans and non-humans (Petney et al. 2007). Despite our lack of understanding of the
178 evolutionary significance of tick-borne pathogens, it is feasible that in areas where ticks are
179 common, defences against this threat may have evolved. In particular, ticks may be more
180 vulnerable to substances applied topically because they are attached to the skin of the host for a
181 prolonged period of time (Carroll et al. 2005). Detailed studies of the biochemistry of
182 Indonesian slow lorises are on-going and may yield further support that slow loris venom
183 contains anti-parasitic properties.
184

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