



## LAPORAN KEMAJUAN (PROGRESS REPORT)

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# **”The only poisonous primate: ecological context & function of slow loris venom”**

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## 1. RESEARCH OBJECTIVES

The aim of the proposed study is to reveal the ecological context and use of venom in slow lorises. The specific objectives addressed are:

1. How toxic are lorises?
2. Do lorises use toxin to kill their prey?
3. Does toxic defence play a part in loris anti-predator strategies?
4. Does toxic defence play a role in reducing loris ectoparasite load?

## 2. DESCRIPTION OF STUDY FIELD

The study will be conducted during eight months at the Ciapus Primate Centre (CPC), located about 6 km west of Bogor on Java in Indonesia. The centre is under the management of International Animal Rescue Indonesia (IARA) that runs a rescue and rehabilitation programme for three species of confiscated Indonesian slow loris (*Nycticebus coucang*, *N. menagensis* and *N. javanicus*). The majority of these lorises are kept in social groups, ranging from one to five individuals, in semi-natural enclosures (ranging in size from 2.0 x 2.0 x 2.5 m to 6 x 6 x 4 m), built around existing vegetation. Each cage is provided with a sun roof, live and dead enrichments as well as places to hide from keepers and conspecifics, and is lit up by night with dim red halogen lights. The lorises are provisioned three times per night with a variety of natural food (mixed fruits, insects, sago, palm weevil, larva, bird eggs etc.).

## 3. RESEARCH MATERIAL OR OBJECTS TO BE INVESTIGATED

Among the very few mammals known to produce toxins, the slow loris of Southeast Asia (*Nycticebus spp.*) is the only primate that appears to be poisonous. The bites from slow lorises are usually delivered after a threatened animal raises its arms tightly above its head, combining secretions of its brachial gland with saliva (Nekaris, Pimley et al. 2007). The bites have been reported to cause oedema and fester in both humans and conspecifics and can take weeks to heal, leave loss of fur and/or deep scarring. In extreme cases, bite recipients

may enter anaphylactic shock, experiencing a burning tongue and throat, a sensation of heat, red itching skin, very low blood pressure, shock, painful muscle convulsion, pain in the heart and kidney region, respiratory problems, heart problems, unconsciousness, or in extreme cases, death ((Wilde 1972; Streicher 2004) Nekaris and Fry, pers. obs.).

The study of the venomous systems of animals, including invertebrates, snakes, lizards and frogs, has provided fascinating insight into their interactions with predators, prey and competitors, as well as has yielded promising medical advances through development of pharmacological agents (Harvey, Bradley et al. 1998). A handful of attempts have already been made to characterize loris toxin. (Alterman 1995) found that slow loris secretions repelled some predators, including a variety of cats, sun bears, and civets. He also demonstrated that the slow loris' procumbent anterior incisors, or toothcomb, normally ascribed dietary intake and grooming functions, is effective as a venom delivery system by conducting liquid upward. Both Krane et al. (2003) and Hagey et al. (2007) (Hagey, Fry et al. 2007) demonstrated that the protein peptides in the brachial oil are homologous with domestic cat Fel-dl peptides. Consequently, they proposed a dual use of the brachial gland, suggesting that the primary uses of loris secretions are to communicate with other lorises, with the toxin being a by-product in some species susceptible to allergens. All of these studies were conducted on a small sample size of captive animals, mostly of unknown taxonomic origin. Therefore the three studies call for further work to elucidate the toxins' actions.

## **4. RESEARCH APPROACH AND METHODS**

### **4.1. How toxic are lorises**

To characterize baseline levels of toxicity of loris venom, we will analyse the chemical components of saliva and gland exudates of slow lorises. Saliva and gland secretion were collected during routine health checks from the lorises kept at the centre (n=96) on a continuous seven week basis. Saliva was collected using plastic syringes or cotton swabs, whereas exudates were obtained using cotton swabs. The urine will be collected from animals in their cages using cotton swabs and/or plastic syringes. Muscle tissue and internal

organs will be sampled from dead specimens, obtained from frozen loris carcasses kept by CPC. All procedures were approved by the Oxford Brookes University Committee for Ethics in Animal Research.

Toxin characterisation of the samples will be done according to previous work of Hagey et al. (2007) and will include: 1) identification of volatile and semi-volatile compounds using a GC-MS and (2) non-volatile compounds using nano ESI-MS, 3) determination of the protein contents of the gland using LC-MS, 4) separation and purification of the toxin using RP-HPLC, 5) Amino acid sequencing, 6) mRNA Extraction, 7) amplification of sequences encoding protein or peptide toxin types, and 8) determination of the three-dimensional protein structures.

#### **4.2. Do lorises use toxin to kill their prey?**

To investigate whether slow lorises use their venom to capture prey, we observed loris prey capture techniques, as well as the behaviour and health of prey post attack. A total of 27 lorises were provided with three different types of prey animals (grasshoppers, geckos, worm meals, larvae and crickets). The observation was done using a head lamp (Petzl® 4.5V, Crolles, France) with red filter. The prey animals used in the study will be captured in the area of the centre or bought from the local market. We used “all occurrences sampling” to register the prey capture behaviour of the slow lorises as well as the behaviour of the bitten prey animals.

#### **4.3. Does toxic defence play a part in loris anti-predator strategies?**

To investigate whether the lorises use their toxin as chemical defence to repel predators, we investigated the behaviour response of captive slow lorises in the presence of potential predators. We conducted the experiments in two standardized test cages, with wire mesh at all sides (width: 2.1m, length: 2.1m, height of left cage: 1.8m, height of right cage: 2.1m), separated from each other by approximately one meter (Figure 1 & 2). Each cage was lit up by night with dim red halogen lights and was provided with a sleeping box, a feeding basket, a drinking bowl, lianas and leaves. At the start of each experiment, consisting of a habituation phase (2-3 days) and an experimental phase (4 days), we took out the subjects

from their home cages and placed them individually in the test cages. When possible, we placed them in the test cages already the night before the habitation phase. After the experimental trials were completed, we returned them to their home cages again. One loris could not be returned to its home cage due to difficulties re-introducing this individual to its previous social group.

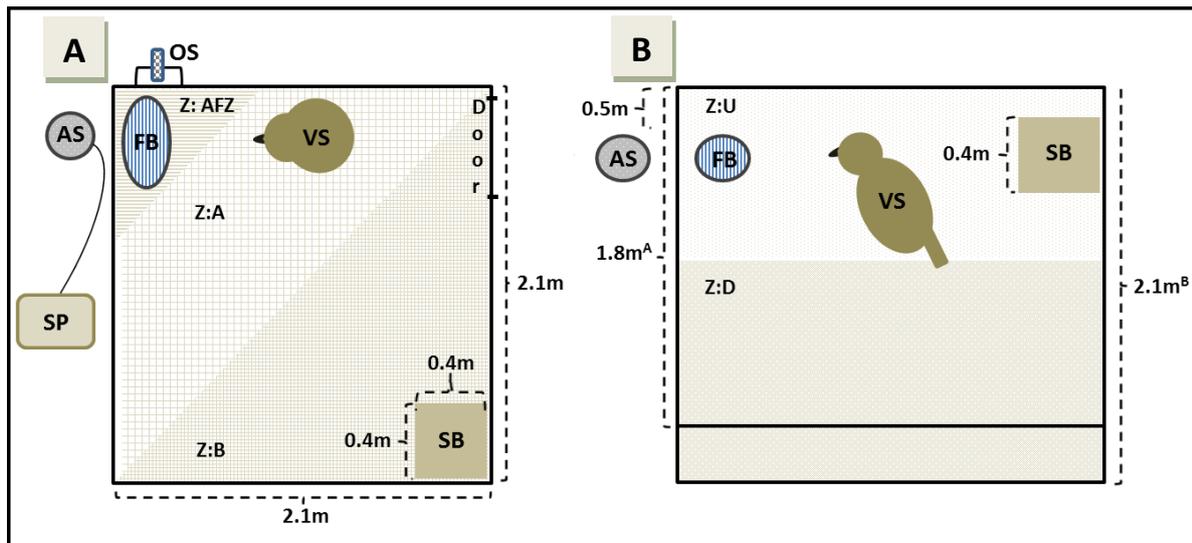


Figure 2. The test cage seen from above (A) and from the side (B) and their measurements (right<sup>A</sup> and left<sup>B</sup> cage); separated in different spatial zones (Z): FZ, A, B, U and D; with placements for acoustic (AS), olfactory (OS) and visual (VS) stimuli; sound recorder (SB), feeding basket (FB) and sleeping box (SB).

Each experimental session started with a minimum of two days of habituation. During the habituation, the procedure followed the experimental set-up of the control trial (see below), to habituate the lorises to the presence of the observer as well as the technicalities. Since the experiment coincided with the feeding of the lorises, and the stimuli would be presented next to the feeding place, we considered the subjects to be habituated when they started to feed within five minutes from the start of the observation. When necessary, we increased the time of the habituation from 30 to 60 minutes. If two days were not enough, we prolonged the habituation phase until our criteria of habituation was fulfilled.

To simulate the presence of reticulated python (*P. reticulatus*), orangutang (*P. pygmaeus*) and crested hawk-eagle (*N. cirrhatus*) in the experimental trials, we presented a combination of olfactory, visual and acoustic stimuli of these predators to the slow lorises (Treves 1998; Table 1). At the start of each trial, which coincided with the approximate time of the routine

food provisioning (6.30pm or 23.00pm), we provided fruit (banana, mango, mangosteen, orange and/or papaya) inside a feeding basket placed in one corner of the cage, to attract the loris to approach an area where we simultaneously placed the stimuli (Blumstein, Ferando et al. 2009; Kappel, Hohenbrink et al. 2011). The feeding baskets were designed by the loris keepers at the centre with the purpose to increase the feeding time of the lorises. Each basket consisted of small parallel bamboo bars, placed widely enough to allow the lorises to get their hands between to reach the fruits, but narrowly enough to not enable fruits to be taken out (Figure 1). During four evenings, we randomly presented the lorises to stimuli from each of the three predators and to one control. Each night, we carried out two experimental trials (one per cage), each lasting for 30 minutes. To minimize the risk that the second tested individual would get exposed to the stimuli presented for the first tested individual, the area where the stimuli were presented in each cage, was positioned on the opposite corner of the two cages. In addition, our first experimental trial per evening always started with the control or the python, both without acoustic stimuli. We made the behavioral observations from a point about 2m away from the test cages, using a head lamp with red filter (Petzl® 4.5V, Crolles, France).

Table 1. Details about the predation stimuli used in the experiments: the common and scientific name of the predators, the combination of stimuli used for each of the predatory species (A: acoustic, O: olfactory and V: visual stimuli), site where the olfactory stimuli were collected.

Common name	Species	Stimuli	Site of collection
Reticulated python	<i>Python reticulatus</i>	O, V	Cikananga WS; Petshop, Bogor
Orangutan	<i>Pongo pygmaeus</i>	A, O	Cikananga WS
Crested hawk-eagle	<i>Nisaetus cirrhatus</i>	A, O, V	Cikananga WS

WS: Wildlife Sanctuary

We used fresh faeces as olfactory stimuli in the experiments, collected from captive animals at Cikananga Wildlife Centre in Java (orangutan, hawk-eagle, python) and from Purnomo pet shop in Bogor, Java (python; Table 1). The reticulated pythons (n = 2, one male, one unknown) and the crested hawk-eagles (n = 12, 5 females, seven males) had been kept on a

carnivorous diet, whereas the orangutans ( $n = 2$ , whereof 1 female and 1 male) on an omnivorous diet. We portioned the faeces into 1-2g portions in 2ml Eppendorf tubes (Hamburg, Germany), and stored them frozen until usage (maximum 13 weeks). About 1-3hr beforehand, we defrosted the faeces to be used in the nightly experimental trials (Kappel et al. 2011). During the experiments, we placed the tubes containing the faeces about 0.2 m from the provisioned food. To avoid inter-specific contamination of diseases or parasites, we placed the olfactory stimuli outside the cage mesh, out of reach for the lorises (Figure 2).

We used self-designed animal models as visual stimuli of reticulated python (length: 1.7m, circumference: 0.23m) and crested hawk-eagle (height: 0.39m, length: 0.52m, chest width: 0.15m; Figure 3). The models were placed inside the test cage, about 0.8m away from the feeding basket (Figure 2). Due to difficulties to design a model to represent the large sized orangutan, we did not use visual stimulus for this predatory species.

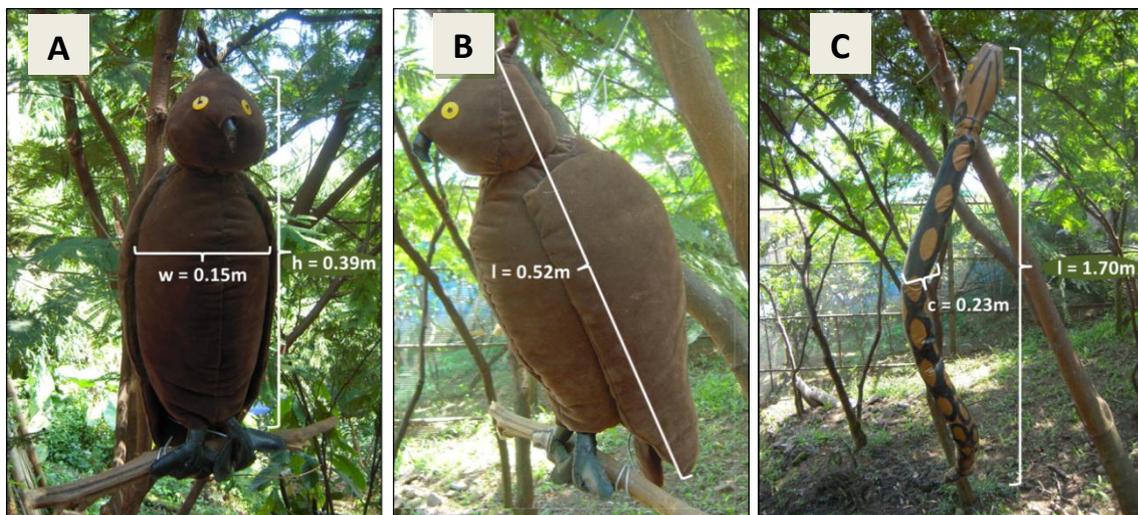


Figure 3. The models of crested hawk-eagle (A & B) and reticulated python (C) used as visual stimuli in the experiments; width ( $w$ ), length ( $l$ ), circumference ( $c$ ).

As acoustic stimuli, we used vocalisations from the hawk-eagle and the orangutan, purchased from the commercially available recordings from Xeno-canto database (<http://www.xeno-canto.org>), and from the Macaulay Library of Natural Sounds (<http://macaulaylibrary.org>; Table 2). We used the Raven Interactive Sound Analysis Software (2003, Cornell Lab of Ornithology, Ithaca, NY, free download), to amplify the

vocalisations to  $30.0 \pm 0.5$  kU (the amplitude unit used by Raven Software), and to extract 13-18 seconds long call sequences. During the 30 minute experimental trials, these sequences were played back, separated by 2-minute sequences without acoustic stimuli. To avoid pseudo-replication we used a total of three different call sequences (one sequence per trial), throughout our experiments (Hurlbert 1984; McGregor 2000). We broadcasted the acoustic stimuli with a portable computer (Lenovo notepad) connected to an active speaker (MiniVox Lite, Anchor), placed about 0.5m away from the feeding place. We made sure to keep the volume constant for all playback experiment.

Table 2. The source, citation and the country of origin of the recordings of *N. cirrhatus* and *P. pygmaeus* used as acoustic stimuli in the experiments.

#	Scientific name	Source	Citation	Country
1	<i>N. cirrhatus</i>	Macaulay library	Philip D Round, ML39823	Thailand
2	<i>N. cirrhatus</i>	Macaulay library	Arnoud B van den Berg , ML70518	Indonesia
3	<i>N. cirrhatus</i>	Xeno-canto, database	David Farrow, XC19651	India
4	<i>P. pygmaeus</i>	Macaulay library	John MacKinnon, ML 82007	Malaysia
5	<i>P. pygmaeus</i>	Macaulay library	John MacKinnon, ML82026	Malaysia
6	<i>P. pygmaeus</i>	Macaulay library	Martjan Lammertink, ML164237	Indonesia

#### **4.4. Does toxic defence play a role in reducing loris ectoparasite load?**

To investigate whether gland secretion, saliva and/or a mixture of gland secretion and saliva (proportion 1:2) from the lorises have a repellent effect on ectoparasites, we will conduct two types of experiments. The first experiment is a two-choice test, where the ectoparasites have the choice between (1) entering a tube coated with one of the three loris fluids leading to a reward, or (2) entering a tube coated with water without reward. A mouse placed in a small cage at the end of the tube, will be used as reward, separated by a piece of fine meshed wire ensuring that the ectoparasites cannot reach the mouse. We will consider a test fluid to have a repellent effect if a significantly reduced number of the ectoparasites enter the tunnel with loris fluid compared to the tunnel with the control.

At the start of each test, we will place hungry ectoparasites in a transparent chamber connected to two 5 cm transparent tubes. The fluids will be placed in the beginning of each tube, about 1 cm from the chamber (Figure 4). By using a syringe, three drops of the fluids will be placed in the tubes via a hole located at the top of the tunnel. If the test fluids are not so volatile and if they act in the gas phase, using the right temperature might be critical (Dautel, personal communication). Thus, to ensure that the test is conducted in a biologically meaningful temperature range, we will keep the tube system in the same temperature as the fur of the lorises. We will consider an experiment completed when the ticks have reached the end of one of the tunnels.

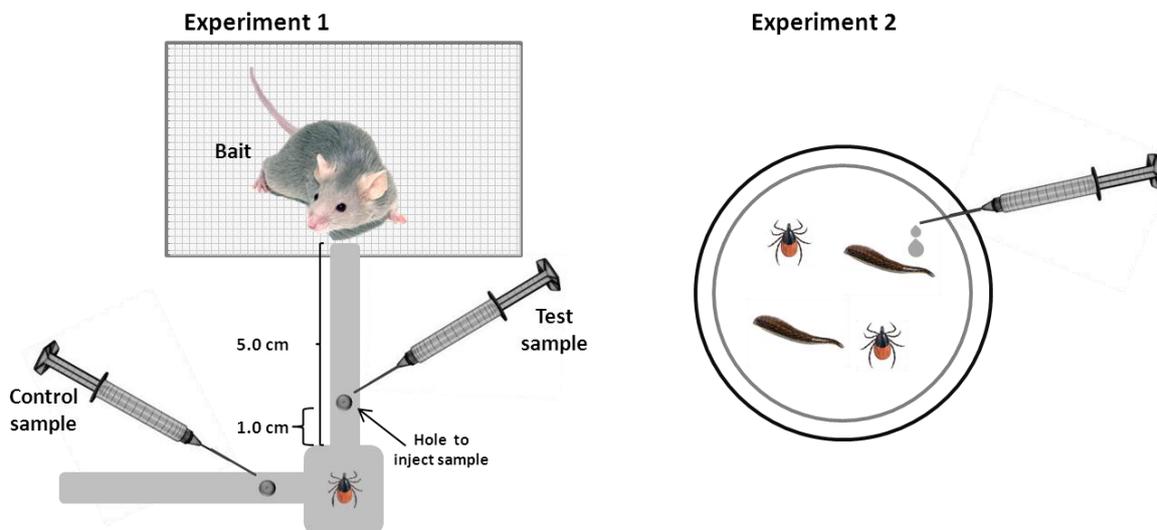


Figure 4. We will conduct a series of three experiments.

The ticks and leeches that we will use in the experiment will be obtained locally from the forest, whereas the mouse will be bought at the local market. We will test a total of 150 ticks and 90 leeches per fluid type in the three different loris species (50 ticks and 30 leeches per species). To minimize the risk for statistical bias in the sample size, we will use fluids from at least five different individuals for each loris species.

In the second experiment, we will evaluate the direct effect of the different test fluids on the ectoparasites. We will place the ectoparasites on a petri dish. By using a syringe, three drops

of the fluids will be applied directly on top of the ectoparasites. We will use all occurrence sampling to record the behavioural and physical response of the ectoparasites. We will conduct the test on a total of 60 ticks and 60 leeches per fluid type in the three different loris species (20 ticks and 20 leeches per species). To minimize potential bias in the experiment, we will use fluids from at least five different individuals for each loris species.

## 5. PRELIMINARY RESULTS

### 5.1. How toxic are lorises

During the first six months of our study, we collected a total number of 213 gland secretion (*N. coucang*: 137, *N. javanicus*: 59, *N. menagensis*: 17) and 166 saliva samples (*N. coucang*: 110, *N. javanicus*: 41, *N. menagensis*: 15; Table 3).

Table 3. The samples of gland secretion and saliva collected from February to September 2012, separated into species, sex and month of collection.

Species	Sex	Gland Secretion					Saliva				
		Feb	April	May	July	Aug	Feb	April	May	July	Aug
<i>N. coucang</i>	F	12	13	17	17	13	5	5	17	17	13
	M	11	14	17	11	12	4	9	17	11	12
<i>N. javanicus</i>	F	5	3	3	2	1	0	1	3	2	1
	M	4	11	14	7	8	0	4	14	7	8
<i>N. menagensis</i>	F	1	3	2	1	0	0	2	2	1	0
	M	1	2	3	2	2	1	2	3	2	2

## 5.2. Do lorises use toxic to kill prey?

To study the prey capture behaviour of the slow lorises, we presented six types of prey animals (cricket, larva, grasshopper, praying mantis, water beetle, gecko) to 26 different individual slow loris (*N. coucang*: 17, *N. Javanicus*: 6, *N. Menagensis*: 3). We observed the slow lorises capture cricket, larva, grasshoppers and praying mantis (Table 2). However, we did not observe them catch neither the water beetle nor the geckos. The majority of the prey animals were instantly killed by getting their heads bitten off. Since the caught prey animals were immediately killed, it was not possible to observe the behaviour and health of prey post attack.

Table 2. The prey animals given and caught by the slow lorises from February to September 2012.

Species	Cricket (# ind)	Grasshopper (# ind)	Meal worm (# ind)	Larva (# ind)	Praying mantis
<i>N. coucang</i>	31 (3)	16 (11)	5 (2)	24 (6)	
<i>N. javanicus</i>		15 (1)			1 (1)
<i>N. menagensis</i>		4 (3)			

# ind: number of individuals

## Does toxic defence play a part in loris anti-predator strategies?

We have conducted predation experiments with a total of 28 slow lorises (*N. coucang*: 16, *N. javanicus*: 12). In total, we registered 6175 behavioural scans in *N. coucang* and 5810 in *N. javanicus*. Throughout the study, we observed only one single individual rubbing its brachial gland towards breast and neck, possibly distributing gland secretion. This was a *N. coucang* that repeated this behaviour seven times while the stimuli of hawk-eagle were presented. Besides, we observed one individual licking the inside of its arm while grooming. This was a single event carried out by a *N. javanicus* in the presence of python stimuli, and we were not able to confirm whether the tongue of the loris was in contact with the actual brachial gland.

## **6. PROBLEMS ENCOUNTERED**

### ***6.1. How toxic are lorises***

During the first three months of data collection of gland secretion and saliva, we obtained fewer saliva samples than expected. During these first three months, we collected the saliva samples using a syringe. Since then, we have used a cotton swab to obtain the saliva samples, and this has been very successful.

### ***6.2. Do lorises use toxic to kill prey?***

As already mentioned above, since the bitten prey animals were immediately killed, it was not possible to observe the behaviour and health of prey post attack.

### ***6.3. Does toxic defence play a part in loris anti-predator strategies?***

The building of the test cages took longer time than we first anticipated. When we took

### ***6.4. Does toxic defence play a role in reducing loris ectoparasite load?***

To be able to test the repellent effect of gland secretion and saliva from the lorises, we first need to collect sufficient samples of these fluids during the routine health checks of the lorises kept at the centre. We did not yet conducted this part of our study.

## **7. PLANNED ACTIVITIES IN THE NEXT THREE MONTHS**

The next few months will be spent back in England to summarize the data that have been collected this far. Currently, we are in the process of applying for export permit to export our gland secretion and saliva samples from Indonesia for analyses in Australia. During the next couple of months, we will continue this process. In the beginning of next year, I plan to come back to Indonesia to conduct the last part of the study, to clarify whether toxic defence play a role in reducing loris ectoparasite load.

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