The species composition and distribution of hematophagous insects collected by light-traps in and near cave systems of Madagascar

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Abstract

A survey of insects, concentrating on blood-sucking Diptera, was conducted in 11 cave systems on Madagascar in the context of a study on hematoparasites of bats roosting in these formations. Flying insects were trapped with CDC light-traps that were simultaneously placed for one night in each cave system from dusk to dawn in three separate positions: 1) outside the cave, mostly in forested areas and in a few cases within degraded natural vegetation, and in close vicinity to the cave entrance; 2) in the entrance zone of the cave, with penetrating sunlight and associated fluctuating temperature; and 3) in the interior (dark zone) of the cave, completely removed from sunlight, and with largely constant temperature. The 1705 collected blood-sucking insects were mainly Culicinae (9.1%), Anophelinae (2.3%), Phlebotominae (75.7%), and Ceratopogonidae (12.8%). Mosquitoes were more common in the exterior traps, with some culicines at entrance traps. Phlebotomine sand flies and Ceratopogonidae biting midges were generally present at all three trap positions, although there were some notable differences in the number of individuals recovered. Some distinct shifts were noted between collection localities situated along a latitudinal (bioclimatic) cline, nearly 1500 km in length, in the relative abundance of the four taxonomic groups in the different cave systems.

Key words: Culicidae, Culicinae, Anophelinae, Phlebotominae, Ceratopogonidae, horizontal distribution, cave ecosystem, relative abundance, potential zoonoses vectors

Résumé détaillé

Les insectes diptères hématophages, tels que les moustiques (Culicidae), les phlébotomes (Psychodidae) et les Ceratogonidae, sont vecteurs de nombreux agents infectieux comme des filaires, des protozoaires, des bactéries et des virus. La plupart des maladies dues à ces agents sont des zoonoses qui affectent les animaux sauvages ou domestiques aussi bien que les Hommes. Pour mieux comprendre les mécanismes de la transmission vectorielle de ces agents infectieux, nous avons réalisé une recherche sur les insectes hématophages, vecteurs avérés ou potentiels, en s'intéressant à leurs distributions et leurs habitats. Cette recherche s'insère dans le contexte d'un vaste programme mené sur les parasites sanguins des chauves-souris à Madagascar, principalement pour les chauves-souris cavernicoles. En pratique, nous avons documenté la distribution des insectes hématophages à proximité et dans les grottes. Au niveau mondial, peu d'informations sont disponibles sur ces distributions, en particulier à Madagascar car l'effort d'échantillonnage réalisé précédemment était orienté vers des études taxonomiques plutôt que vers la distribution et l'écologie des vecteurs.

Cette recherche a été menée dans trois zones bioclimatiques de Madagascar (sèche – Massif de l'Ankarana dans l'extrême Nord-ouest, Région d'Anjohibe près de Mahajanga, et Massif du Bemaraha dans le Centre-ouest ; subaride – autour de Toliara, dont Ankililoaka, Tsimanampetsotsa, et Itampolo; et subhumide – Hautes Terres centrales dont la forêt d'Ambohitantely). Dans chacun de ces sites, nous avons mis en place une procédure d'échantillonnage pour collecter la faune entomologique nocturne, en se concentrant sur les insectes Diptera hématophages volant activement dans différentes portions des écosystèmes de grottes et à proximité de ces habitats. Au total, 11 systèmes de grottes ont été échantillonnés à Madagascar, conjointement pour les insectes et pour les chauves-souris. Des pièges lumineux CDC (modèle 2836BQ, BioQuip Products, Rancho Dominguez, CA, USA) équipés d'une ampoule ordinaire à incandescence ont simultanément fonctionné aux trois emplacements

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suivants : 1) à l'extérieur des grottes mais non loin de l'entrée, ordinairement en zone de forêt, parfois en zone de végétation naturelle dégradée ; 2) à l'entrée de la grotte, dans la zone soumise aux fluctuations nycthémérales de la lumière du jour et de la température ; et 3) à l'intérieur de la grotte où l'obscurité est permanente et la température pratiquement constante.

Les échantillons d'insectes de chaque piège ont été conservés séparément dans de l'éthanol 90 - 95 %, dans des tubes appropriés, fermés et étiquetés. Ces échantillons ont été envoyés à Montpellier, France, et triés par Vincent Robert en divers entités taxonomiques avec la plus grande précision possible pour les insectes hématophages. Nous avons concentré les analyses sur trois groupes de Diptera: Culicidae, incluant les sous-familles Culicinae et Anophelinae; Psychodidae, spécifiquement la sous-famille Phlebotominae ; et Ceratopogonidae. Aucun autre insecte hématophage connu de Madagascar, telles que Simuliidae, Tabanidae, Hippoboscidae, Pulicidae et Hemiptera n'a été observé dans ces collectes.

Au total, les 1705 insectes hématophages se répartissaient en Culicinae (9,1 %), Anophelinae (2,3 %), Phlebotominae (75,7 %) et Ceratopogonidae (12,8 %). Ces différents groupes d'insectes ne se répartissaient pas de façon homogène entre les trois emplacements examinés, à l'extérieur, à l'entrée et à l'intérieur des grottes. Des différences d'abondance entre les divers groupes ont aussi été notées entre les sites des trois domaines bioclimatiques. Les moustiques ont surtout été abondants à l'extérieur des grottes, avec quelques Culicinae dans les pièges à l'entrée des grottes. Les phlébotomes et les Ceratopogonidae ont généralement été présents aux trois emplacements avec des différences dans les abondances relatives. De plus, d'importantes variations d'abondance ont été notées dans les collectes réalisées à plusieurs entrées d'un même système de grotte. Enfin, des tendances ont été notées en fonction d'un gradient latitudinal (bioclimatique) sur une distance de près de 1500 km, pour l'abondance des quatre groupes taxonomiques dans les divers systèmes de grottes.

Le seul appareil de capture utilisé dans cette étude, pour nombre de raisons logistiques, a été le piège CDC le plus simple, muni d'une lampe à incandescence. La collecte d'insectes est connue pour être dépendante des systèmes de piégeages utilisés. Aussi, il est pratiquement certain que d'autres méthodes de collecte, utilisant par exemple des pièges appâtés par des animaux ou d'autres types de piège CDC avec chaleur infrarouge ou attractants chimiques, auraient procuré des résultats différents. A l'avenir, d'autres suivis devraient utiliser, dans la mesure du possible, plusieurs types de pièges, pendant une période plus longue, et inclure des visites répétées dans les mêmes sites. De telles études seraient à même de tester la robustesse des tendances observées dans la présente étude.

Mots clés : Culicidae, Culicinae, Anophelinae, Phlebotominae, Ceratopogonidae, distribution horizontale, écosystème cavernicole, abondance relative, vecteurs potentiels de zoonoses

Introduction

Mosquitoes (Diptera: Culicidae) and other blood sucking insects, such as phlebotomine sand flies (Diptera: Psychodidae) and biting midges (Diptera: Ceratopogonidae), are known vectors of a wide variety of disease agents, ranging from filaria, blood-borne protozoan parasites, bacteria, and viruses (Lehane, 2005; Cohuet *et al*., 2012). Many of these diseases are important zoonoses in wild animals, as well as having epidemiological implications for humans. In order to understand aspects associated with the circulation of these diseases, notably in specific ecological or geographical zones, it is critical to know aspects of the distribution and habitat preferences of documented or suspected hematophagous insects implicated in their transmission.

In the context of a project investigating blood and filaria parasites of Malagasy bats, for the most part involving species that make their day roosts in caves, we were interested in understanding the distribution of hematophagous insects within and in the immediate vicinity of such underground systems. While considerable research has been conducted on different non-hematophagous arthropods occurring in cave ecosystems around the world (Quate, 1962; Peck *et al*., 1998; Greenslade, 2002; Graening *et al*., 2006; Lavoie *et al*., 2007), few studies have focused on cave-dwelling mosquitoes (Adam, 1965; Vattier-Bernard, 1971), phlebotomine sand flies (Williams, 1976; Leng, 1987; Polseela *et al*., 2007; Apiwathnasorn *et al*., 2011), biting midges (Wirth & Hubert, 1989), or in general local communities of hematophagous insects, which was the principal foci of this current study. Further, concerning Madagascar, virtually nothing is known about the occurrence of these groups of insects in caves. The exception being phlebotomine sand flies, for which several new species to science have recently been described (Randrianambinintsoa, 2013; Randrianambinintsoa *et al*., 2013); however, aspects of their ecological preferences and distribution remain to be elaborated. The horizontal distribution and movements of these different vectors or potential vectors in and out of cave ecosystems are important in deciphering pathogen transmission pathways amongst cave day-roosting bats, as well as other locally occurring vertebrates.

We implemented in different bioclimatic areas of Madagascar a sampling procedure to collect quantitatively the local volant and nocturnal entomological fauna living in different portions of cave ecosystems and nearby habitats outside the cave, and the entomological results are presented herein. As we are particularly interested in host-vector relationships of bat blood parasites, we focus on flying hematophagous dipterans. Our results provide insight into the ecological stratification of these different insects and form the basis for future studies on vectors and mechanisms of disease transmission in cave-dwelling bats.

Materials and methods

Our sampling sites on Madagascar were predominantly in the dry western half of the island and specifically in lowland areas with limestone landscapes, where caves are a marked aspect of the local environment (Table 1). In such karstic zones, the density and diversity of bats are notably high (Goodman, 2011; Goodman *et al*., 2005). While there is some geographical clustering of sampled sites, the majority of which fall along a latitudinal gradient, which bisects several bioclimatic zones (*sensu* Cornet, 1974) (Figure 1). One site is included herein (Ambohitantely) from the Central Highlands, which is located in a more mesic ecosystem and at 1550 m elevation.

Insects were trapped with CDC Miniature Light Traps, employing incandescent light (model 2836BQ, BioQuip Products, Rancho Dominguez, CA, USA). In the vicinity of each sampled cave, traps were simultaneously in operation for one night of capture (dusk to dawn) in three separate positions: 1) outside the cave (**exterior**), often in forested areas or those

Table 1. Different cave sites incorporated into this study. The localities are arranged from north to south. See Figure 1 for geographical position of study sites.

Site	Date	Coordinates	Elevation	Bioclimatic zone (see Figure 1)	Habitat
Grotte d’Andrafiabe, Ankarana	15 Sept 2012	49.0550°E, -12.9417°S	50 m	Dry	Dry deciduous forest on karst.
Grotte des Chauves-souris, Ankarana	14 Sept 2012	49.1181°E, -12.9563°S	120 m	Dry	Dry deciduous forest on karst.
Grotte d’Ambahibe, Ankarana	13 Sept 2012	49.1193°E, -12.9683°S,	80 m	Dry	Dry deciduous forest on karst.
Grotte des Crocodiles, Ankarana	17 Sept 2012	49.0069°E, -13.0111°S	35 m	Dry	Dry deciduous forest on karst.
Grotte d’Anjohibe, north entrance	07 Sept 2012	46.8860°E, -15.5382°S	100 m	Dry	Open anthropogenic woodland on karst.
Grotte de Anjohibe, south entrance	08 Sept 2012	46.8853°E, -15.4249°S	100 m	Dry	Open anthropogenic woodland on karst.
Grotte d’Anjohikely, entrance 1	06 Sept 2012	46.8742°E, -15.5609°S	100 m	Dry	Open anthropogenic woodland on karst.
Grotte d’Anjohikely, entrance 2	09 Sept 2012	46.8775’E, 15.5589’S	110 m	Dry	Open anthropogenic woodland on karst.
Grotte des Chauves-souris, Ambohitantely[1]	20 Sept 2012	47.2892°E, -18.1811°S	1550 m	Subhumid	Montane humid forest.
Grotte d’Anjohikinakina, Bekopaka	6 Nov 2012	44.7677°E, -19.0098°S	100 m	Dry	Dry deciduous forest on karst.
Grotte de Bekoaky, Ankililoaka	10 Feb 2012	43.72267°E, -22.7730°S	80 m	Subarid	Heavily degraded dry deciduous forest on karst.
Grotte d’Andranoilovy, Tsimanampetsotsa	21 Apr 2012	43.7500°E, -24.0500°S	50 m	Subarid	Dry spiny bush on karst.
Grotte de Nikotse, Itampolo[2]	25 Apr 2012	43.94909°E, -24.63848°S	5 m	Subarid	Slightly degraded dry spiny bush on karst.

[1] As explained in the Materials and Methods section, this site is more of a rock shelter than a cave and the “interior” trap set was exposed to outside ambient light and daily fluctuations in temperature.

[2] As explained in the Materials and Methods section, this site is a classical limestone cave, but the deepest accessible section is close to the entrance and the “interior” trap set was exposed to outside ambient light and daily fluctuations in temperature.

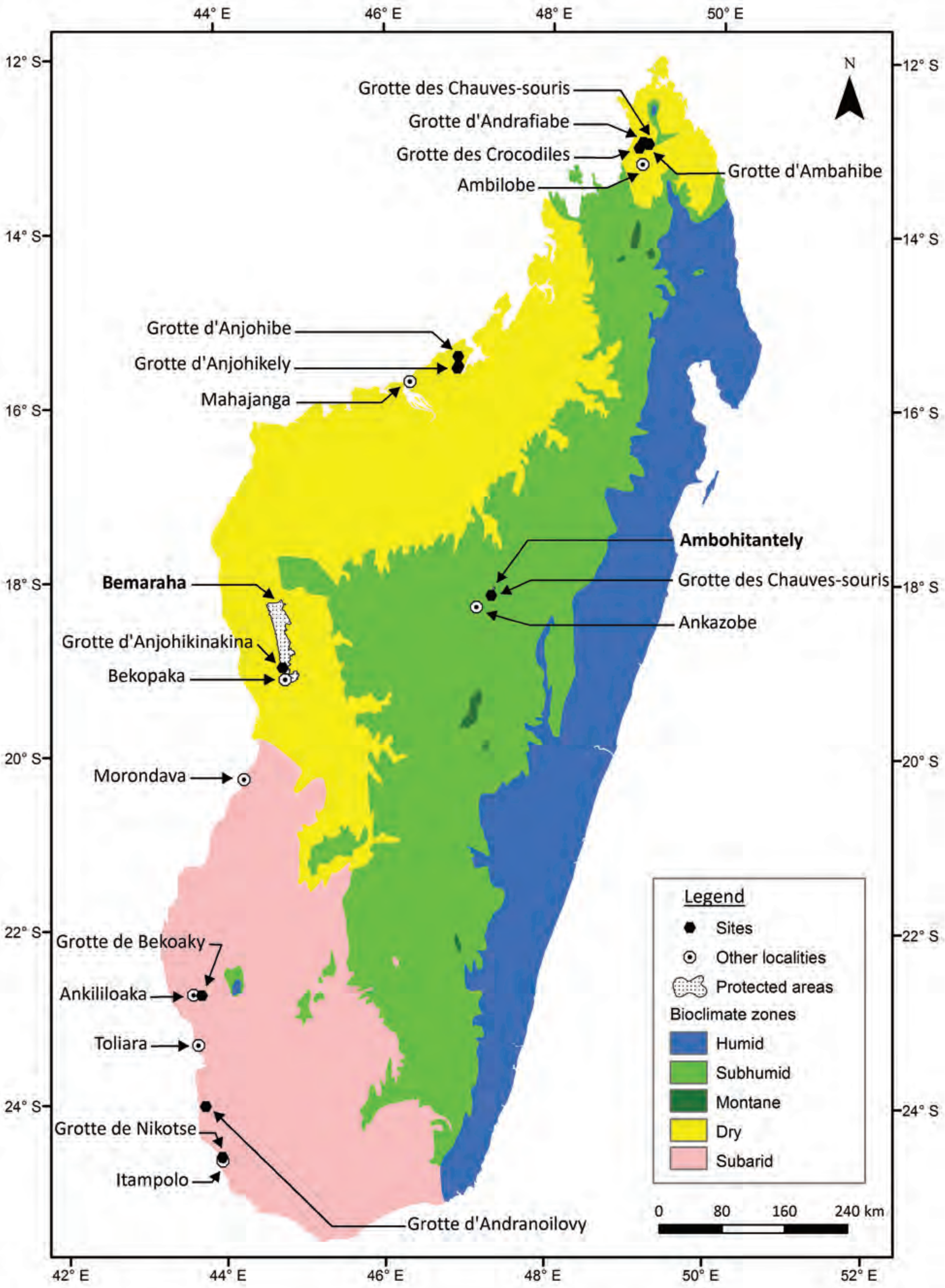

Figure 1. Map of the different cave study sites where CDC light-traps were installed in the context of this study. At each site, three different placements were used: exterior to the cave, interior to the cave entrance and in the interior dark zone. The map overlay is the simplified bioclimatic zones developed by Schatz (2000) after the original classification proposed by Cornet (1974).

with degraded natural vegetation, and within 30 to 50 m of the cave entrance; 2) within the **entrance** zone of the cave, with filtrating sunlight and associated fluctuating temperatures, and 10 to 20 m inwards from the physical opening, depending on structural aspects; and 3) within the dark zone (**interior**) of the cave, completely removed from sunlight and with largely constant temperatures.

Some variants occur at certain sampling sites. In one case (Grotte de Bekoaky), a trap was also in operation in the interior of a cave during external daylight hours, specifically to have a sense of "diurnally" active hematophagous insects that would be present in caves during the roosting period of bats. Further, two sites (Grotte d'Anjohibe, Grotte d'Anjohikely) had repeated sampling on consecutive nights at different cave entrances within the same system to examine potential variability in numbers of captured insects and associated taxonomic representation.

The light-traps set in the interior of the cave were used to capture flying insects attracted by light, keeping in mind that troglobites ordinarily show extreme adaptation to cave environments, including the loss of eyesight and, thus, do not respond to light-traps. The assumption is that hematophagous insects obtained in the cave interior are trogloxenes (Barr, 1963) and complete their life cycle in the zones near the entrance or external environments. The traps placed at cave entrances were presumed to capture insects passing freely to the exterior or potentially finding refuge in the shaded and temperate environments portion of the cave opening.

Insect samples from each light-trap were conserved separately in closed and appropriately labelled vials containing 90-95% ethanol. These samples were then sent to the lab of VR in Montpellier, France, where the insects were separated into different taxonomic assemblages and those associated with hematophagous groups were identified to the most precise taxonomic level possible. Some samples of Phlebotominae sand flies were retained in France and included in on-going and completed taxonomic revisions of Malagasy region members of this family (Randrianambinintsoa, 2013). The balance of the hematophagous insect samples were subsequently transferred to the Centre de Recherche et de Veille sur les maladies émergentes dans l'Océan Indien (CRVOI) laboratory on La Réunion (France) where the individual specimens will be tested with molecular tools as reservoirs or vectors for hematoparasites already isolated from bat hosts captured in each respective cave system. The data presented herein provide entomological insights into the ecological distribution of different potential vectors and a foundation for future research on the collected hematophagous insects.

A series of chi-square association tests were used to determine whether or not trap placement (three sites), geography (11 sampled sites), and hematophagous insects (four study groups) present significant dependency on the effective. Some Pearson chi-square tests (asymptotic) were employed in other comparisons and associated probabilities are presented in cases when test requirements are satisfied (Dytham, 2011; Mehta & Patel, 2011).

Results

General aspects

In total, 11 different sites distributed along a latitudinal cline of nearly 1500 km in length are included in the analysis presented herein. These are located in three bioclimatic zones (Figure 1): 1) **Dry** – four sites in the Ankarana Massif in the extreme northwest (Grotte d'Andrafiabe, Grotte des Chauves-souris, Grotte d'Ambahibe, Grotte des Crocodiles), two sites in the Anjohibe Region, near Mahajanga, in the central northwest (Grotte d'Anjohibe, Grotte d'Anjohikely), one site near Bekopaka, Bemaraha Massif, in the central west (Grotte d'Anjohikinakina); **Subarid** – three sites in the extreme southwest, Toliara area, one near Ankililoaka (Grotte de Bekoaky), one near Tsimanampetsotsa (Grotte d'Andranoilovy), and the third near Itampolo (Grotte de Nikotse); 3) **Subhumid** – one Central Highland site near Ankazobe and within the Ambohitantely complex (Grotte des Chauves-souris).

Both the Grotte des Chauves-souris (Ambohitantely) and the Grotte de Nikotse had particular physiogeography aspects: the first was carved out by ground water and more of a rock shelter than a cave and the latter was a shallow classical limestone cave. In both cases, the "interior" trap set was not in the zone of the cave completely cut off from external light sources and fluctuating daily temperatures.

The different hematophagous insects identified from the collected material at each site are presented in Table 2. As previously mentioned, the insect samples collected in the light-traps were conserved in ethanol. This style of preservation, as compared to pinned specimens, resulted in the dislodgement of insect scales, which in turn created some identification difficulties, particularly for mosquitoes and Lepidoptera. This aspect together with the lack

Table 2. Summary of different hematophagous insects identified from the CDC light-trap samples for the sites included in this study. Details on identified mosquitoes of the subfamilies Culicinae and Anophelinae are given.

Localities	Trap placement	Culicinae	Anophelinae	Phlebotominae	Ceratopogonidae	Corethrellidae	Total	Details on Culicinae	Details on Anophelinae
Grotte d'Andrafiabe, Ankarana	Exterior (forest)	0	17	29	7		53	15 *Uranotaenia*, 2 *Culex* (♂, ♀)	
	Entrance	2	1	420	3		426	2 *Uranotaenia*	*Anopheles pauliani* ♀
	Interior	0	0	23	4		27		
Grotte des Chauves-souris, Ankarana	Exterior (forest)	6	1	39	1	17	64	*Uranotaenia* ♂	*Anopheles pauliani* 2 ♀♀, 4 ♂♂
	Entrance	0	0	35	2		37		
	Interior	0	0	44	1		45		
Grotte d'Ambahibe, Ankarana	Exterior (forest)	0	1	7	3		11		
	Entrance	31	0	30	0		61		
	Interior	0	0	17	0		17		
Grotte des Crocodiles, Ankarana	Exterior (forest)	7	1	45	20		73	7 *Aedes* ♀♀	*Anopheles funestus* ♀
	Entrance	0	0	3	0		3		
	Interior	0	0	26	25		51		
Grotte d'Anjohibe, north entrance	Exterior (forest)	2	1	4	0		7	2 *Mansonia* ♀♀	Unidentified Anophelinae ♀
	Entrance	0	0	58	3		61		
	Interior	0	0	37	8		45		
Grotte d'Anjohibe, south entrance	Exterior (forest)	2	0	0	0		2	*Uranotaenia* ♀, *Culex* (*Culex*) ♂	
	Entrance	0	0	5	3		8		
	Interior	0	0	14	0		14		
Grotte d'Anjohikely, entrance 1	Exterior (forest)	0	0	1	0		1		
	Entrance	0	0	3	0		3		
	Interior	0	0	11	1		12		
Grotte d'Anjohikely, entrance 2	Exterior (forest)	1	0	0	3		4	*Mansonia* ♂	
	Entrance	1	1	142	1		145	Unidentified Culicinae ♀	Unidentified Anophelinae ♀
	Interior	1	0	12	2		15	*Aedes* ♀	
Grotte des Chauves-souris, Ambohitantely	Exterior (forest)	14	0	1	10		25	13 *Uranotaenia* ♀♀, *Orthopodomia* ♂	
	Entrance	0	0	0	0		0		
	Interior	21	0	1	15		37	19 *Uranotaenia* ♀♀, ♀ of another genus	
Grotte d'Anjohikinakina, Bekopaka	Exterior (forest)	15	0	64	31	1	111	unidentified Culicinae 14 ♀♀, ♂	
	Entrance	13	0	58	8		79	10 *Uranotaenia* ♀♀, *Aedes* (*Aedimorphus*) ♂, 2 *Culex* ♀♀	

Table 2. continued

Localities	Trap placement	Culicinae	Anophelinae	Phlebotominae	Ceratopogonidae	Corethrellidae	Total	Details on Culicinae	Details on Anophelinae
	Interior	3	0	3	0		6	2 ♀♀, 1 ♂ *Aedes* (*Aedimorphus*)	
Grotte de Bekoaky, Ankililoaka	Exterior (distrubed forest)	0	0	0	1		1		
	Entrance	1	0	3	1		5	*Orthopodomyia* ♂(?)	
	Interior (day)	0	0	25	0		25		
	Interior (night)	0	0	47	0		47		
Grotte d'Andranoilovy, Tsimanampetsotsa	Exterior (forest)	17	11	1	55		84	*Aedes albocephalus* ♂, *Aedes* (*Aedilorphus*) ♂, 3 unidentified ♀♀	*Anopheles pauliani* 2♀♀, 11 ♂♂
	Entrance	3	4	20	0		27	Aedes	Anopheles pauliani
	Interior	3	1	8	0		12	*Coquilletidia* ♀, Culex ♀, *Aedes* ♀ (abdomen swollen with blood)	*Anopheles pauliani* ♀
Grotte de Nikotse, Itampolo	Exterior (forest)	1	0	7	2		10	*Aedes* ♀	
	Entrance	2	0	1	7		10	*Culex* (*Culex*) ♂	
	Interior	10	0	46	2		58	*Culex giganteus* ♀, *Uranotaenia* ♀, *Culex* (*Culex*) ♂	
Total		**156**	**39**	**1290**	**219**	**18**	**1722**		

of taxonomic resolution at the species level for most Malagasy hematophagous insects did not allow the majority of specimens to be identified to levels more precise than family or sub-family. The exceptions are for the culicidian fauna, with identifications, in some cases, to genus or species (Table 2). Specimens preserved in the manner used herein are ideal for Phlebotominae sand flies and research is currently underway to describe the material resulting from these collections.

We concentrate our analyses on three groups of blood-sucking Diptera (Nematocera): Culicidae, including the subfamilies Culicinae and Anophelinae; Psychodidae, specifically the subfamily Phlebotominae; and Ceratopogonidae. Over 1705 individuals were collected of these three families (Table 3). In addition, 18 specimens belonging to the known hematophagous family Corethrellidae, or frog-biting midges, were identified from the samples (Table 2). However, as these flies feed on the blood of anourans, they were not the focus of this study (Robert & Borkent, 2014). No material of other blood-sucking dipterans known from Madagascar, such as Simuliidae, Tabanidae, Hippoboscidae, and blood-sucking Hemiptera were represented in the light-trap samples. Further, ectoparasites including Siphonaptera, Phtiraptera, Nycteribiidae, and Streblidae were not identified in the unidentified insect collections reported upon herein.

Variation in hematophagous insect groups and trap positions at a given cave site

On the basis of our light-trap captures in different positions relative to cave openings (Table 2), the different groups of hematophagous insects are not equally distributed across the zone ranging from the external environment of each cave system to the dark interior. For example, at Ankarana, falling within the dry bioclimatic zone (Figure 1), in the Grotte d'Andrafiabe (Ankarana) anopheline mosquitoes were common in the exterior trap, rare in the entrance trap, and not present in the interior trap. Further, at the other three sites sampled at Ankarana, members of this mosquito subfamily were only rarely found in exterior traps. In contrast, culicine mosquitoes had a tendency to be more common in entrance positions, as compared to exterior positions, and completely absent in the interior positions.

Phlebotomine sand flies were present at all three trap positions in the four caves sampled at Ankarana, although there were some notable differences in the number of individuals captured. For example, 420

Table 3. Number of identified individuals of hematophagous insects belonging to four different subfamilies or families obtained in the three trapping positions at different localities.

Localities	Culicinae	Anophelinae	Phlebotominae	Ceratopogonidae	Total
Ankarana (n = 4)					
Exterior (forest)	13	20	120	31	**184**
Entrance	33	1	488	5	**527**
Interior	0	0	110	30	**140**
Anjohibe (n = 2)[1]					
Exterior (forest)	5	1	5	3	**14**
Entrance	1	1	208	7	**217**
Interior	1	0	74	11	**86**
Central Highlands (n = 1)					
Exterior (forest)	14	0	1	10	**25**
Entrance	0	0	0	0	**0**
Interior	21	0	1	15	**37**
Bemaraha (n = 1)					
Exterior (forest)	15	0	64	31	**110**
Entrance	13	0	58	8	**79**
Interior	3	0	3	0	**6**
Toliara region (n = 3)					
Exterior (forest)	18	11	8	58	**95**
Entrance	6	4	25	8	**43**
Interior	13	1	126	2	**142**
Total	**156**	**39**	**1291**	**219**	**1705**

[1] Two different caves were sampled, with traps placed at two different portions within each system.

individuals were obtained in the entrance position in the Grotte d'Andrafiabe, while only three individuals were trapped in the entrance position in the Grotte des Crocodiles. For the Ceratopogonidae, they were present in all four caves sampled at Ankarana, and generally in all three trap positions within a cave; the number of collected individuals was variable, ranging from zero (exterior and entrance positions) to 25 (interior position). These data indicate that Phlebotominae and Ceratogonidae flies occur in considerable numbers in the dark zone of caves. Future taxonomic work on the collected material of these two subfamilies will verify if the species occurring at the three sampled sites in each cave are the same or there are some ecological differences in their horizontal distributions. As all four sites at Ankarana were sampled during the same period (mid-September 2012), the differences in the distribution of each hematophagous insect group between different caves and measures of relative abundance do not reflect seasonal variation, but some inherent dissimilarity between these cave systems, which might be associated with a number of parameters (e.g. local ecological conditions, proximity of bat roosts).

At sites in the extreme southwest falling within the subarid bioclimatic zone (Figure 1), an area of Madagascar with notably higher daily maximum temperatures and a longer dry season as compared to sampled sites further to the north (such as Ankarana), patterns in hematophagous insects were different. Culicine mosquitoes were found in both the Grotte d'Andranoilovy and the Grotte de Nikotse, in all three light-trap positions and within the former cave anopheline mosquitoes were also found at the interior site. In the Grotte de Nikotse, phlebotomine sand flies were more common in the interior placed trap than those at exterior and entrance positions. In this regard, as mentioned earlier, it is important to mention that the deepest accessible portion of Grotte de Nikotse was not out of the light zone and the portion of the cave near the interior trap set was distinctly cooler than the entrance zone. Hence, different groups of hematophagous insects could find refuge in the "interior" portion of this cave some 10-15 m from the opening, which did not fall within the dark zone.

On the basis of data presented in Table 3, there is a statistically significant difference between the effective of each family of blood-sucking insect and trap placement (Pearson chi-square, $\chi^2 = 339.0$, d.f. = 6, $P < 0.001$). In terms of total composition, Phlebotominae was always more abundant at each grouped locality (Table 3), whereas Anophelinae and Culicinae were notably less common.

Variability within a cave system

In the context of our light-trap captures, two cave sites (Grotte d'Anjohibe, Grotte d'Anjohikely) had repeated sampling, but in each case at different entrances within each cave system (Table 2). At Anjohibe, the north and south cave entrances, both surrounding by small islands of vegetation, are separate by about 450 m ground distance. A large colony of the fruit bat *Rousettus madagascariensis* occurs in relatively close proximity to the north entrance. For mosquitoes, the results are very similar for the two sites, with few individuals being captured. At the north entrance, Phlebotominae and Ceratopogonidae flies were distinctly more common at all trap positions than at the south entrance. These two sites were sampled on consecutive nights and the differences are associated with local variation rather than seasonal conditions.

At Anjohikely, entrance 1 was surrounded by a small island of vegetation and entrance 2 by denuded vegetation. No mosquitoes were identified from the entrance 1 site and a limited number from the entrance 2 site, which included single Culicinae from each of the three trap positions and a single Anophelinae from the entrance trap position. As at Anjohibe, there were considerable differences in the number of phlebotomine sand flies captured at the two sampled zones of Anjohikely; for example, at the two entrance trap sets numbers ranged from three individuals (entrance 1) to 142 individuals (entrance 2). For ceratopogonids, a single individual was trapped at the entrance 1 site (in the interior position), while a few individuals were captured at all three trap positions at the entrance 2 site. Hence, as at Anjohibe, some differences were noted in the presence and abundance of hematophagous insects associated with different entrances within the same cave system.

Variability between "nocturnal" and "diurnal" trapping

At the Grotte de Bekoaky, the trap in the interior position was operated during two different periods (Table 2): external day and external night. During these two periods, not a single individual belonging to the families Culicidae or Ceratopogonidae was obtained. For phlebotomine flies, 25 individuals were captured during the day period and 47 during the night period. Hence, the results do not demonstrate an important difference in circulating hematophagous insects during these two periods, with the possible exception that phlebotomine flies may have been slightly more active during the night, perhaps associated with the nocturnal exodus or predawn entry of day-roosting bats.

Abundance and variation of various insect groups in different geographical zones and associated trap positions

The trap capture data for the different sites have been combined into five different areas based on geographical proximity (Table 3) and correspond to different bioclimatic zones on Madagascar or in the case of the dry bioclimatic zone falling out along a broad latitudinal cline (Cornet, 1974; Schatz, 2000).

Mosquitoes

Mosquitoes were relatively uncommon amongst the identified hematophagous insects captured in the light-traps. In total, 156 culicines and 39 anophelines were found, representing 9.1% and 2.3% of blood-sucking insects.

At Ankarana, Culicinae mosquitoes were more commonly trapped at cave entrances, as compared to exterior to the cave, while Anophelinae mosquitoes showed the opposite pattern between these two trap positions. In contrast, neither subfamily of mosquito was captured in the interior of the four sampled caves. The number of mosquitoes captured at Anjohibe was notably limited, but one of seven captured Culicinae was in the cave interior. In contrast, in the Toliara region, Culicinae and Anophelinae mosquitoes were relatively common in exterior traps, less common in entrance traps, and present in interior positions, with the former subfamily being more common than the latter. The presence of mosquitoes in the interior positions of the Toliara region caves is in part an artefact of the structure of the Grotte de Nikotse, which is not a deep cave (see Materials and Methods, Table 1). At Ambohitantely, in the Central Highlands, anophelines were not captured in any trap position, and culicines were more common in the exterior and entrance positions, as compared to the interior position. The physical situation of the Ambohitantely cave site can explain this observation (see Materials and Methods, Table 1). In general, between the five geographical zones, there was a distinct tendency for mosquitoes to be most common either at the exterior and entrance sites, and rare or absent from the interior sites.

Phlebotomine sand flies

Members of the subfamily Phlebotominae were the most commonly identified hematophagous insects in the samples. This pattern held for the five geographical zones. In total, 1291 individuals were obtained, which accounts for 75.7% of the different blood-sucking insects in these respective collections.

Amongst the combined Ankarana sites, members of this subfamily were captured in similar numbers at exterior and interior trap positions and were distinctly more common at entrance trap positions. In contrast, at the Anjohibe and Anjohikely sites, phlebotomine flies were notably uncommon at exterior sites, common at interior sites, and distinctly more abundant at entrance sites. At the Toliara region sites, there was increasing abundance of sand flies passing inwards within the cave systems from the exterior to interior sites. Finally, in the Central Highlands, few individuals were captured at the exterior and interior traps sites and the vast majority at the entrance site. Thus, the distribution of members of this group of flies between the five different geographical zones showed different patterns of abundance relative to the three different trap positions.

Biting midges

Members of the family Ceratopogonidae were in general not abundant amongst the hematophagous insects in the light-trap samples; they were present in all five combined geographical zones. In total, 219 individuals were obtained, which accounted for 12.8% of the different blood-sucking insects in the light-trap collections.

Members of this family were equally common at exterior and interior trap locations in Ankarana, and distinctly less common at entrance trap sites. In contrast, at the Anjohibe and Anjohikely sites, they were never common, but there was a tendency of increasing number of captured individuals passing along the horizontal cline from exterior to interior sites. At the three sampled caves in the Toliara region, these insects were distinctly more common at exterior traps sites than those at entrance and interior sites. At Ambohitantely, biting midges were found at exterior and entrance sites, in nearly equal numbers, but absent from the interior site. In conclusion, between the five different geographical zones, ceratopogonids flies to large extant showed different patterns of horizontal distribution between the three different trapping positions.

Correlations to latitudinal clines and bioclimatic zones

A chi-square analysis between the taxonomic composition of blood sucking insects represented at the five grouped sites (Table 3) showed statistically significant differences (Pearson chi-square, χ^2 = 397.1, d.f. = 12, $P < 0.001$), which in turn confirms biogeographical differences in the number of individuals captured of each family. The five grouped sites segregated in three bioclimatic zones (Figure 1), each of which has particular meteorological characteristics, often with clinal latitudinal variation. It is important to mention that within the dry climatic zone covering much of western Madagascar, there are, for example, important levels of decreasing rainfall from north to south. The number of blood-sucking insects capture in the Central Highlands rock shelter, Grotte des Chauves-souris (Ambohitantely), is notably low, which is probably correlated with annual low average temperatures and greater rainfall.

Discussion

The three families of Diptera which form the principal focus of this study are known across their distributions as vectors of protozoan blood parasites (Culicidae, Psychodidae, Ceratopogonidae), viruses (Culicidae, Psychodidae, Ceratopogonidae), rickettsia and bacteria (Culicidae, Psychodidae), and nematodes and filaria (Culicidae, Ceratopogonidae) (Kettle, 1995; Lehane, 2005). On Madagascar, the role different members of these families have in transmitting various diseases to cave roosting bats still remains to be determined, but relatively high rates of hematoparasites and filaria have been recorded amongst the bats occupying the same caves surveyed with the CDC light-traps (Ramasindrazana *et al*., unpublished data). The intent of ongoing research, employing molecular tools and using the hematophagous insect samples collected in the context of this current study, will be to correlate which taxa are reservoirs and presumed vectors of zoonoses identified from the bats. Further, systematic studies on this material will provide precise details regarding the species limits of the phlebotomine and ceratopogonids flies in our light-trap samples.

The single collection device used in our study was a CDC light trap model fitted with an incandescent bulb. The use of other techniques for hematophagous insects, such as animal-baited traps or infrared heat, or fragrance strips with octenol, lactic acid, and CO_2, may have produced different results. However, for a variety of logistical reasons we were limited to the use of CDC traps. This trap type with UV light tube, as compared to incandescent light, have proven in the Seychelles to be more attractive to Culicine mosquitoes (Le Goff *et al*., 2012). Future studies should use, when possible, a wider range of trap types, over a longer period of sampling, and include repeated visits to the same site. Such work can test the robustness of the patterns found in this current study.

Caves are presumed to be excellent resting places for a variety of different insects associated with the more stable temperatures and levels of humidity, as compared to outside these systems. Two important aspects remain to be resolved associated with cave environments for mosquitoes: 1) access for females to obtain a blood meal, for which we suspect that bats play an important role, but this remains to be conclusively demonstrated, and 2) access to stagnant or slow flowing water for females to lay eggs and such sites were absent from the specific zones of caves we sampled, except Grotte d'Andrafiabe, Grotte des Chauves-souris (Ankarana), Grotte des Crocodiles, Grotte des Chauves-Souris (Ambohitantely), and Grotte d'Andranoilovy. On continental Africa, mosquitoes have been found breeding in isolated water pools deep within cave environments (van Someren & Mutinga, 1971).

In the case of phlebotomine sand flies on Madagascar, aspects of their natural history are very poorly known; for example, no specimen at the preimaginal stage has been collected and no troglobitic species (strictly cave dwelling) is known. In caves from tropical Central Africa, strictly cave-dwelling phlebotomine sand flies are closely associated with bat day-roost sites and, depending on the taxa, larval stages develop in guano or rock substrates (Vattier-Bernard, 1971). The eyes of these sand flies have atrophied and their integument lacks pigmentation, attesting to their being troglobites.

Caves can in fact be complex systems with some notable and subtle differences in a variety of parameters that have a direct relationship to different abiotic variables, such as air movement, fluctuating temperatures, and relative humidity (Culver & Pipan, 2009). On Madagascar, these factors have an important impact on the choice of day roost sites for bats and caves with greater internal complexity tend to have more diverse bat species assemblages (Cardiff, 2006); this same general pattern has been found elsewhere in the world (Dwyer, 1971; McDonald *et al*., 1990; Brunet & Medellín, 2001; Rodriguez-Duran &

Soto-Centeno, 2003). These factors also presumably have important influence on the horizontal distribution of hematophagous insects. For example, in the current study we found that the Malagasy culicine fauna demonstrates a clear preference for zones outside of caves and in entrance areas, as compared to the dark interior areas. In contrast, phlebotomine and ceratopogonid flies occur in different areas along this horizontal distribution.

In an analysis of blood parasites of 14 species of cave-dwelling bats on Madagascar, Raharimanga *et al*. (2003) found general prevalence rates of 21% and the parasites included Haemoproteidae, microfilaria, and *Trypanosoma*. If the transmission of these parasites to bats was indeed via hematophagous insects, the identity of the vector(s) remain(s) unknown on Madagascar. In contrast, elsewhere in the Old World, Haemoproteidae and microfilaria are suspected to be transmitted by the dipteran families Culicidae and Ceratopogonidae, while *Trypanosoma* by Siphonaptera or other dipterans, such as Hippoboscidae, Nycteribiidae, and Streblidae (Euzéby, 2008). It is important to underline that these proposed vectors and modes of transmission remain to be demonstrated.

Of the 93 bats found positive in the Raharimanga *et al*. (2003) study, 92 belonged to the genera *Miniopterus* (Miniopteridae) and *Myotis* (Vespertilionidae) that tend to make their day roost sites relatively close to cave entrance areas or at least in the zone that receives some measurable level of external light. Hence, these bat taxa are in the infiltration range of Culicinae mosquitoes into caves from the external environment. In contrast, bat species that roost in the interior portion of the same cave systems were completely or largely free of hematoparasites; this would seem to indicate they are out of the transmission shadow of mosquitoes. However, the situation is presumably not that simple, with the possibility that Phlebotominae and Ceratopogonidae flies, which circulate deep into caves, being vectors of bat blood parasites. Perhaps there is a phylogenetic component to certain levels of immunity to different hematoparasites amongst cave-dwelling bats, such as the families Pteropodidae, Hipposideridae, and Molossidae, which were almost unanimously negative in the study of Raharimanga *et al*. (2003). Further, research on presumed and known vectors of hematoparasites, their distribution in cave systems, as well as detailed laboratory investigations of syntopic bats, should help to resolve some of these questions.

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