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IMPACT OF DEFORESTATION ON RODENT DISTRIBUTION AND ON THE PREVALENCE OF LEPTOSPIROSIS IN CAMBODIA

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PART 1 : RESUME EN FRANÇAIS

PART 1: RESUME EN FRANCAIS

1. Introduction

Le taux de déforestation au Cambodge se classe parmi les plus élevés du Sud Est de l'Asie et du Monde. La conversion des forêts en cultures agricoles entraîne des changements brusques et irréversibles de ces écosystèmes. De tels changements peuvent modifier la distribution des rongeurs et donc des pathogènes qu'ils hébergent. Entre autres, la leptospirose représente un problème de santé majeur au Cambodge où elle est endémique. L'hypothèse posée dans cette étude est que la déforestation modifie la population des rongeurs et, par conséquent, la transmission de leptospires.

La leptospirose est une zoonose mondialement répartie. La maladie est due à des espèces de leptospires pathogènes, bactéries appartenant à l'ordre des *Spirochetacae* et du genre *Leptospira*. Ces bactéries peuvent infecter tous les mammifères dont l'Homme. Certains serovars de leptospires démontrent une spécificité d'hôte. En particulier, les rongeurs sont considérés comme des réservoirs majeurs de leptospires pathogènes pour l'Homme. L'infection est maintenue chez les animaux infectés par une colonisation des tubules rénaux par les leptospires, qui sont ensuite relâchées dans l'environnement dans leurs urines. La plupart des espèces de leptospires survivent dans un environnement aqueux ou dans un sol humide. La présence de ces leptospires pathogènes dans l'environnement constitue ainsi le principal mode de contamination de l'Homme : par contact avec un environnement souillé d'urine d'animaux infectés. Dans les pays tropicaux où la leptospirose est endémique, la riziculture, l'élevage, les pluies abondantes ou encore la présence de rats dans les habitations ont été identifiés comme des facteurs de risque de la maladie. La circulation de leptospires pathogènes pour l'Homme a été mise en évidence chez les rongeurs en Asie du Sud-Est, en particulier chez des espèces synanthropiques mais également chez des espèces forestières peu étudiées.

Les taux élevés de déforestation en Asie du Sud-Est coïncident avec une augmentation d'émergence de maladies infectieuses. Un certain nombre d'études sur les maladies infectieuses émergentes s'accorde à établir qu'un des mécanismes d'émergence est la modification de l'habitat par l'homme. Les modifications de l'habitat par l'Homme peuvent impacter négativement l'intégrité de l'écosystème et la biodiversité. Ces changements dans la structure de l'écosystème peuvent également conduire à des modifications du système hôte-pathogène. Ainsi les modifications de l'habitat ont le potentiel de modifier la dynamique d'une maladie directement ou indirectement en modifiant l'abondance, la démographie, le comportement, la

réponse immune ou encore le contact entre espèces et la composition des espèces réservoirs (Gottdenker et al., 2014).

En effet, la modification des paysages par l'Homme se traduit par une perte de l'habitat des espèces forestières et sa fragmentation. Cela peut entraîner des conséquences potentielles sur la transmission des agents infectieux tels que l'augmentation de la densité d'une espèce réservoir de l'agent pathogène ; l'augmentation de contact interspécifique, c'est-à-dire entre des espèces différentes et en particulier en mettant en contact la faune sauvage avec l'homme directement ou indirectement avec les animaux domestiques ; une augmentation des contacts intra-spécifiques (au sein d'une même espèce), due à la fragmentation de l'habitat et au regroupement des ressources (Brearley et al., 2013). Ce sont des exemples de mécanismes hypothétiques par lesquels la modification de l'habitat peut aboutir à une modification de la prévalence des maladies. Dans le cas des agents infectieux transmis par les rongeurs, des études sur les hantavirus décrivent une telle modification (Suzán, Marcé, et al., 2008 ; Blasdell et al., 2016). Ces virus se transmettent par contact direct avec des fluides corporels infectés entre rongeurs et peuvent infecter l'Homme. Des épidémies à hantavirus étaient survenues dans un contexte de modification de l'habitat, où la biodiversité était réduite. L'explication proposée par ces articles était que la modification de l'habitat favorisait des espèces de rongeur opportunistes qui étaient réservoirs du virus et qui en l'absence de compétition, pouvaient atteindre des densités élevées conduisant à une augmentation de contacts intra-spécifique et consécutivement à une augmentation de la prévalence (Suzán, Marcé, et al., 2008). Il est donc important de déterminer quels sont les agents pathogènes infectieux transmis par les rongeurs qui pourraient suivre la même dynamique, suite à la perturbation de l'habitat.

Peu d'études se sont intéressées à l'impact de modification d'habitat sur la leptospirose, mais montrent une corrélation entre les espèces de leptospires et la topographie (Ivanova et al., 2012). En particulier, une étude suppose que l'infection des rongeurs par *Leptospira* spp. serait corrélée à la fragmentation de l'habitat forestier (Morand et al., 2015).

La conversion de forêt en zone agricole n'est pas seulement le passage d'un écosystème à un autre, c'est un processus progressif dans le temps. Pour comprendre les changements dans la transmission d'agents pathogènes zoonotiques dus à la déforestation, il est nécessaire de s'intéresser à l'enchaînement complexe des événements qui interviennent pendant cette période transitoire, ou chronotone. Le chronotone, terme proposé et défini par (Bradley, 2004), est « *l'interface temporelle entre deux types de paysage* », « *c'est une période relativement rapide*

de transformation qui sépare deux types d'utilisation prolongée des terres ». C'est cette période transitoire qui représente un risque pour la santé publique et qui est étudiée dans cette thèse.

2. Méthode

L'étude se base sur un design de « space-for-time substitution» dans lequel le gradient géographique de déforestation dans un même site est appréhendé comme une substitution à la dynamique temporelle de la déforestation, le chronotone. Trois zones, correspondant à trois étapes de la déforestation, sont définies : « **Forêt intacte** » (Forêt indemne d'activité de déboisement ou d'activité faible) ; « **Forêt perturbée** » (Forêt où l'activité de déboisement est intense) et « **Plantation récente** » (Champs agricoles divers : rizière sèche, plantations de cassava ou de maïs, moins de un an après le début de la déforestation). Ce modèle de chronoséquence pose l'hypothèse que ces trois zones correspondent à une même zone à trois temps différents au cours du processus de déforestation. Pour respecter cette hypothèse, les trois zones sont choisies géographiquement proches les unes des autres au sein de chaque site et ayant moins d'un an depuis le début de la déforestation, afin de limiter les différences spatio-temporelles autres que celles dues au processus de déforestation. L'étude a été réalisée dans cinq sites au Cambodge dans les provinces du Mondulkiri et de Kampong Thom et fut répétée en saison des pluies et en saison sèche pour ces cinq mêmes sites. Les rongeurs ont été capturés simultanément dans les trois zones d'un même site pendant huit nuits consécutives. Les rongeurs capturés sont prélevés, marqués avec une boucle auriculaire unique et relâchés à leur lieu de capture. Les espèces de rongeurs ont été déterminées par marqueur moléculaire et séquençage (barcoding).

La première partie des résultats de cette thèse s'intéresse aux effets de la déforestation sur la communauté de rongeur. Les mesures de composition, richesse et diversité d'espèce, ont été comparés entre les trois zones. Le modèle de capture-marquage-recapture a permis d'estimer la densité de population de rongeur dans les trois habitats.

La deuxième partie des résultats porte sur les taux d'infections de la leptospirose chez les rongeurs capturés. L'infection par *Leptospira* spp a été testée par PCR en temps réel. Le gène *rrs*, universellement présent chez les leptospires, a été amplifié afin de détecter les infections par des espèces pathogénes et intermédiaires (PCR1). Le gène *LipL32*, présent uniquement chez les leptospires pathogènes, a permis de détecter les infections par des leptospires pathogènes uniquement (PCR2). Les leptospires pathogènes détectées ont été séquencées.

3. Résultats

A l'issu des deux saisons de captures, 522 rongeurs ont été capturés et pu être identifiés. Onze espèces ont pu être déterminées par marqueur moléculaire. *Mus cervicolor Rattus* sp *R3* et *Maxomys surifer* étant les espèces les plus fréquemment capturées et représentent 95% du total d'animaux capturés.

3.1. Déforestation et rongeurs

Les indices de diversité et de richesse d'espèce ressortent significativement différents entre les trois zones de déforestation au sein de chaque site. La zone correspondant à la forêt perturbée contenait le plus grand nombre d'espèces de rongeurs et une plus grande diversité d'espèces que la forêt intacte et les champs récents. La richesse et diversité des espèces de rongeur augmentent donc de façon transitoire pendant la déforestation, puis diminuent après conversion agricole.

La densité de rongeur augmente également au cours de la déforestation, avec des valeurs significativement plus élevées en forêt perturbée qu'en forêt intacte. La densité de rongeurs dans les zones agricoles récentes apparaît significativement dépendante de la saison, atteignant des valeurs élevées (de 15 à 48 animaux par hectare) en saison des pluies mais des valeurs faibles en saison sèche.

Les analyses statistiques d'écologie (analyse de correspondance et matrice de dissimilarité) ont montré que la déforestation s'accompagnait d'un changement progressif de composition d'espèce de rongeur. Certaines espèces sont capturées exclusivement en forêt intacte (*Rattus andamanensis*¸ *Leopoldamys sabanus, Berylmys bowersi*) supposant leur disparition avec la déforestation, bien que le faible effectif de capture de ces espèces ne permette pas de conclure. En revanche, des espèces forestières telles que *Maxomys surifer*, *Berylmys berdmorei* et *Niviventer fulvescens* sont également capturées dans les zones perturbées indiquant une persistance de ces espèces malgré le déboisement. Aucune espèce forestière ne persiste à la fin de la déforestation et à sa conversion en champs agricoles (à l'exception de *Rattus* sp *R3* qui fut capturé dans toutes les zones). La population de rongeurs dans les champs est alors caractérisée par très peu d'espèces (montré par des indices de richesse et diversité faibles) dominés par une espèce majoritaire *Mus cervicolor* et une densité élevée.

Des espèces agricoles, notamment *Mus cervicolor,* étaient également présentes dans les forêts perturbées indiquant une invasion précoce, exacerbée pendant la saison sèche. En effet, les dissimiliarités de composition entre la forêt perturbée et les champs agricoles récents sont

minimes pendant la saison sèche. Les conditions difficiles thermiques et l'absence de nourriture dans les champs en saison sèche peuvent expliquer une migration des espèces agricoles vers la forêt alentour. La forêt perturbée se compose donc à la fois d'espèces forestières et d'espèces agricoles qui n'étaient jusque-là pas en contact.

3.2. Déforestation et *Leptospira* spp.

L'infection par des espèces pathogènes et intermédiaires de *Leptospira* spp a été mise en évidence chez sept espèces de rongeurs avec des prévalences variant de 1 à 100%. Les valeurs extrêmes étant expliquées par des effectifs très faibles d'animaux pour certaines espèces. La prévalence totale chez les rongeurs était de 13.5% en saison de pluies et 7.3% en saison sèche mais la différence entre les deux saisons n'était pas significative à l'analyse multivariée.
La prévalence n'était pas significativement différente entre les espèces ni entre les trois niveaux de déforestation. Les rongeurs mâles étaient significativement plus à risque d'être infectés par des espèces pathogènes et intermédiaires de *Leptospira* spp.
L'infection par des leptospires pathogènes uniquement n'a été trouvée que chez trois espèces : *Mus cervicolor* capturés dans les champs en saison des pluies et *Maxomys surifer* en forêt perturbée et *Berylmys bowersi* en forêt intacte en saison sèche. Les prévalences respectives chez ces espèces étaient de 4.5% (12/267), 3.8% (1/26) et 50% (1/2).
L'isolement et le séquençage des leptospires pathogènes chez ces animaux infectés a permis l'identification de trois espèces de leptospires pathogènes pour l'Homme : *L. borgpetersenii*, *L. weilii* et *L. interrogans*.

4. Discussion

Les résultats de cette étude montrent une modification complète de la composition d'espèces de rongeur, engendrée par la déforestation. Les indices de richesse et de diversité d'espèces augmentent transitoirement pendant la déforestation pour atteindre des minimums dans les champs récemment convertis à l'agriculture. La déforestation et conversion semblent favoriser une espèce : *Mus cervicolor*, espèce majoritaire dans les champs et qui atteint des densités élevées. La présence de cette espèce dans les zones en cours de déforestation indique une invasion précoce de cette espèce (moins de un an après le début de la déforestation).
L'infection par *L. borgpetersenii* chez *Mus musculus* fait d'elle une espèce à risque. Cette leptospire nécessite une transmission directe, contrairement aux autres espèces de leptospires

qui ont la capacité de survivre dans un environnement humide et se transmettent principalement par contact indirect. La transmission de cette leptospire peut donc dépendre de la proportion d'animaux infectés dans la population mais également de la densité de sa population d'hôte. Les fluctuations de densité et de migration de cette espèce, induites par les saisons et exacerbées par les pratiques agricoles peuvent donc modifier la dynamique de la leptospirose.
L'infection par *L. borgpetersenii* d'un *Maxomys surifer* capturé en forêt perturbée pendant la saison sèche où se retrouve préférentiellement *Mus cervicolor,* pourrait indiquer un passage de leptospire d'une espèce agricole hôte à une espèce forestière. L'infection d'un animal seulement ne nous permet pas de conclure mais permet toutefois de soulever l'hypothèse que l'assemblage transitoire d'espèces en forêts perturbées, en augmentant la probabilité de contact entre espèces différentes pourrait entraîner un potentiel changement d'hôte des leptospires.

5. Conclusion.

La leptospirose est un problème de Santé Publique majeur au Cambodge. Bien qu'endémique dans ce pays et d'incidence supposée élevée, la leptospirose est négligée car sous-diagnostiquée et souvent confondue pour des cas de paludisme et de dengue, majoritairement présents dans le pays. Elle y est peu étudiée et les facteurs de risques de transmission à l'Homme ne sont pas identifiés au Cambodge.
Les résultats de cette thèse ont permis d'identifier quelles étaient les modifications écologiques qui survenaient au cours de la déforestation. Ainsi, la déforestation aboutit à une réduction de la diversité et richesse d'espèces de rongeurs, et favorise l'introduction et la persistance d'une espèce *Mus cervicolor*. Les résultats sur la leptospirose ne montraient pas de modification de la prévalence au cours de la déforestation, ni entre les espèces de rongeur. Toutefois les leptospires pathogènes séquencées correspondaient à trois espèces différentes dans les trois stades de déforestation.
La présence de leptospires zoonotiques dans les trois niveaux de déforestation indique un potentiel risque de leptospirose humaine associé à des activités forestières et agricoles ou encore la consommation de rongeurs sauvages. Il est donc nécessaire de déterminer par la suite le risque associé à chacune de ces activités.

PART 2: LITERATURE REVIEW

PART 2: LITERATURE REVIEW

1. DEFORESTATION IN CAMBODIA

Home to some of Southeast Asia's oldest and most diverse forests, Cambodia is a recognized hotspot of biodiversity but is also a place of massive deforestation and of emerging infectious disease (Myers et al., 2000 ; Morand et al., 2014).

The rate of deforestation in Cambodia has accelerated in the last few years and has now reached about 208,000 hectares a year (Forest, 2015). The proportion of primary forest, characterized by naturally regenerated forest of native species without any ecological disturbance or any visible human activities, decreased to barely 3% of total forest cover in Cambodia in 2015.

All presented data on deforestation in Cambodia are excerpted from a Forest Trend Report from 2015 (Forest, 2015). Cambodian forest is increasingly converted to other land use, with rubber plantations the most common plantation type, followed by sugar, pulp and paper plantations and then cassava and rice fields. These conversions are enabled by economic land concessions, though the legality of these land allocations is questionable. By 2015, 2.2 million hectares, twelve percent of the whole country had been allocated to economic land concessions (ELC) with no legal framework to justify these allocations. Tragically, they often overlap with protected areas and in 2013, 14 percent of protected forest land had been allocated resulting in even more loss of evergreen and primary forest (Forest, 2015). Aggravating the situation, the collection of timber on ELC, by becoming the main source of wood harvested in Cambodia, is believed to facilitate illegal logging in nearby areas, exacerbating the disputably legal logging from land concessions (Forest, 2015). There is a concerning lack of effective regulatory framework regarding land conversion and logging in Cambodia that will inevitably lead to tremendous loss of forest ecosystem and biodiversity (Wilcove et al., 2013). The link between forests and human health has been highlighted on several aspects, from the known role of forest in improving the human environment (for example by absorbing airborne pollution), its role as a source of bioactive medicinal compounds (Skirycz et al., 2016), to a recently explored causal link between forest change and emergence of infectious diseases which often originate in animals (Wilcox, Ellis, 2006). Understanding the relationship between deforestation and its potential impact on human health could help promote responsible forest management and control of forest-linked disease to lessen the impact.

Deforestation, by disrupting the natural environment of rodent species, will affect the species distribution and their densities and could thus have important consequences on the pathogens they carry. Rodents have been implicated in the emergence and spread of infectious diseases of importance to human health such as plague, murine typhus, scrub typhus, leptospirosis and hantavirus haemorrhagic fever, etc...(Herbreteau et al., 2012) Among them, leptospirosis, represents a major threat to public health in South-East Asia.

2. LEPTOSPIROSIS REVIEW

Leptospirosis is a worldwide zoonosis caused by pathogenic bacteria, the leptospires that are transmitted directly or indirectly from animals to humans.

2.1. *Leptospira* biology

Leptospires are bacteria that belong to the *Spirochetacae* order, *Leptospiraceae* family and *Leptospira* genus. Leptospires are thin, highly motile, slow-growing spirochetes, about 0.1-0.3µm in diameter by 6–20µm in length (Levett, 2001). Too thin to be visible under the ordinary microscope, dark-field microscopy is most often used to observe leptospires after staining (figure 1).

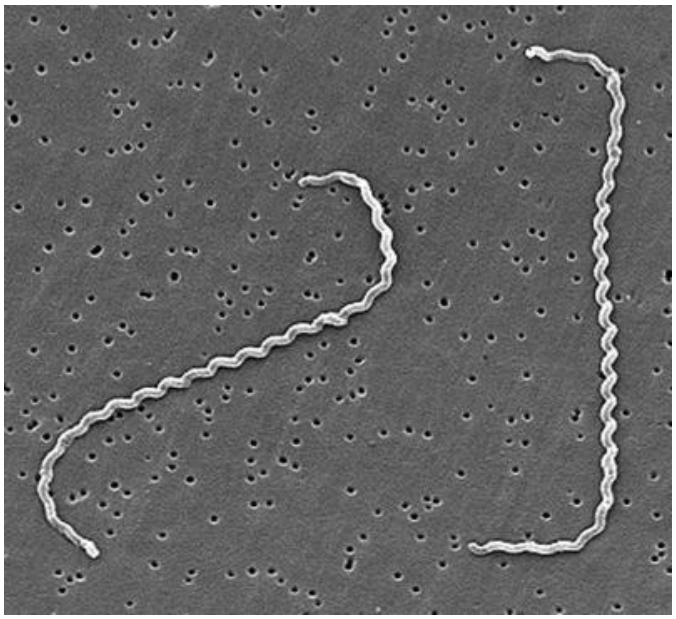

FIGURE 1 *Scanning electron micrograph of L. interrogans serovar icterohaemorrhagiae strain RGA*
SOURCE : Levett (2001)

They can be distinguished from other bacteria on the basis of their unique helical shape and the presence of periplasmic flagella. Leptospires have distinctive hooked ends. Two periplasmic flagella with polar insertions are located in the periplasmic space and are responsible for motility. Leptospires can exhibit two distinct forms of movement, translational and nontranslational that enable them to move in aqueous media (Goldstein, Charon, 1988).

Leptospires have a typical double membrane structure in common with other spirochetes, in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlain by an outer membrane. They are obligate aerobes with an optimum growth temperature of 28–30°C. Leptospires are catalase and oxidase positive. Leptospiral lipopolysaccharide has a composition similar to that of other gram-negative bacteria, but has lower endotoxic activity (Levett, 2001).

2.2. *Leptospira* classification

Leptospira family contains both pathogenic leptospires, having the potential to cause disease in animals and humans and saprophytic leptospires that are free living bacteria in wet environment and generally considered not to cause disease.

Initially, two serological species were recognized, namely pathogenic *Leptospira interrogans* and saprophytic *Leptospira biflexa.* Both complexes (*L. interrogans* and *L. biflexa*) have been divided into several serovars using the cross-agglutinin adsorption test and antigenically related serovars were grouped into serogroups. The *Leptospira* classification was historically based on serological characteristics and comprised over 260 pathogenic serovars (See Appendix 1).

Recent genetic research has resulted in the reclassification of *Leptospira* spp., on the basis of DNA relatedness and has led to 12 pathogenic species and 5 saprophytic species (Bharti, 2003). However, there are still many new species that are believed to exist and yet to be discovered.

The two classification systems based on the serovar and genetic concepts do not correspond as strains belonging to the same serovar may belong to different *Leptospira* species (Appendix 2). However, both the antigenic and genetic classification systems are in common use (Morey et al., 2006 ; Levett, 2001).

Currently, twenty *Leptospira* species have been identified on classical DNA-DNA hybridization studies and 16S ribosomal RNA gene phylogeny (Xu et al., 2016). They comprised of pathogenic, intermediate and saprophytic groups as detailed on the phylogenetic tree (Figure 2). The intermediate group consists of five species that occasionally cause disease in humans and animals. The six saprophytic species are not pathogenic and among them, *L. biflexa* is a soil bacterium, unable to replicate intracellularly.

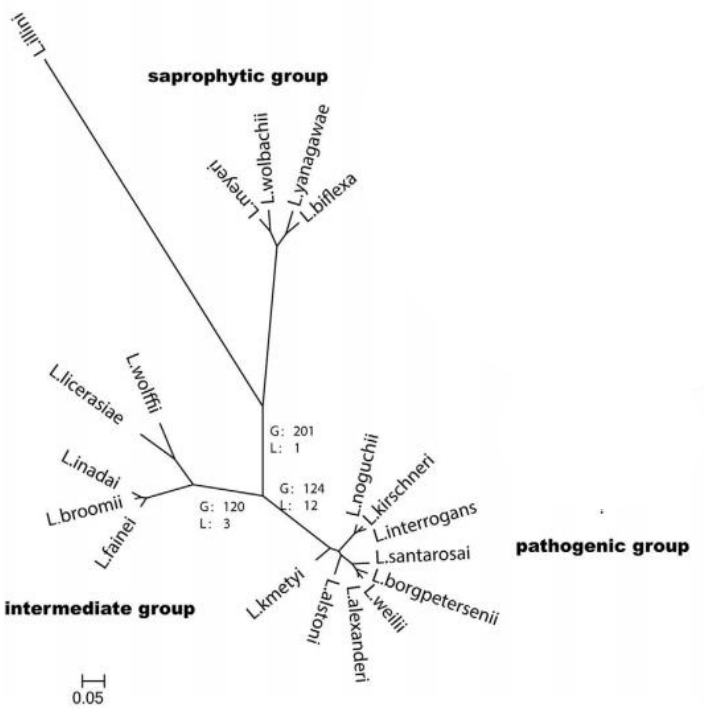

FIGURE 2 *Phylogenetic tree of Leptospira species*

Phylogenetic analysis based on the maximum likelihood of the concatenated core genes of the Leptospira genome with Leptonema illini as the outgroup. Scale bar indicated an evolutionary distance of 0.05 amino acid substitutions per position.

SOURCE : XU et al. 2016.

Recent genomic and phylogenetic studies of *Leptospira* spp. supported that the actual biodiversity of *Leptospira* spp inferred that host adaptation might be the driving force of *Leptospira* diversification and evolution (Xue et al., 2008 ; Xu et al., 2016 ; Ko et al., 2009).

Complete genome sequencing of different *Leptospira* species showed a high diversity among species and found a range of genome size diversity higher than any other zoonotic pathogens (Xu et al., 2016). This high genomic variability was attributed to massive gene gain and loss events that allowed for adaptation to specific niche conditions and changing host environments (Xue et al., 2008). Saprophytic species are closer to the most recent common ancestor while intermediate and pathogenic species formed the two deepest branches, suggesting that virulent genes favoring host infection have been acquired during the evolution of the genus. Loss of genes involved in metabolic pathways and gains of virulent genes, for example these responsible for motility and chemotaxis required to colonize and invade a host, could thus explain the evolution from strains capable of surviving in complex ambient environments into those adapted for pathogenic life (Xu et al., 2016).

2.3. Host species

Leptospirosis affects both humans and animals and has been reported in most mammal groups worldwide (Levett, 2001).

Leptospira has been found in many mammal species worldwide: from common animals (rodents, canidae, bovidae…) to more exotic discoveries (racoon, polar bear, whales) (Duncan, 2012 ; Grune Loffler et al., 2015 ; Richardson, 2003 ; Jabłoński, 2016 ; Junge et al., 2007).

Some serovars are commonly associated with particular animal reservoirs. Certain host-serovar specificity exhibits relatively high fidelity, for example *Rattus* species and serovar Icterohaemorrhagiae, and mice with serogroup Ballum serovars (Bharti, 2003). More examples are given in Appendice 3.

Leptospires are usually adapted to their primary hosts and cause little illness in these. Clinical signs can be seen when a different serovar is introducer to a host species and the symptoms are variable, depending on the serovar or *Leptospira* species, the host and the animal immune system.

Leptospirosis in dogs can be asymptomatic or range from a transient fever to an acute, fulminant illness with fever, anorexia, vomiting, liver and renal failure. Four syndromes were described in canine leptospirosis: icteric, hemorrhagic, uremic and reproductive syndromes (Faine et al., 1999).

Cattle are the maintenance host for hardjo-bovis, infection with this serovar will often produce a carrier state in the kidneys associated with long-term urinary shedding. In addition, infections with hardjo-bovis can persist in the reproductive tract. Many leptospiral infections in cattle are subclinical, particularly in nonpregnant and nonlactating animals. In cattle and pig, clinical signs of leptospirosis are mostly reproductive symptoms such as abortion, mummified fetus, fertility loss, agalaxia... In horses, leptospirosis can result in chronic recurrent uveitis. During the healing phase, animals can be asymptomatic carrier of leptospires. Present in the renal tubules for short to long period of times, leptospires are excreted into the environment in the urine (see Figure 3).

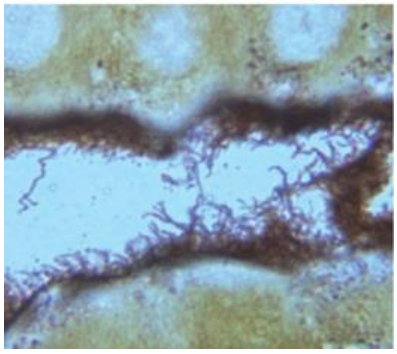

FIGURE 3 *Photomicrograph of a Warthin-Starry stained section of kidney tissue from sewer rat. Leptospires are seen as silver-impregnated filamentous structures within the proximal renal tubule lumen (400x magnification)*
SOURCE : Ko et al., 2009.

Animal reservoirs that may pose a risk for human exposure include livestock, dogs (Mgode et al., 2015) but also wildlife: raccoons, prairie dogs (Olds et al., 2015 ; Richardson, 2003) and rodents.

Rodents are considered a major reservoir of human leptospirosis as they can be asymptomatic carriers of the bacteria and may continually excrete leptospires into the environment throughout their life (Faine et al., 1999). Leptospirosis is maintained by persistent *Leptospira* colonisation of the proximal renal tubules of infected animals (Figure 3) who can thus shed the bacteria in their urine and discharge them into the environment intermittently, regularly for months or years, or even for their lifetime in the case of rodents (Faine et al., 1999).

2.4. Environmental reservoir

Once excreted in the urine into the environment, leptospires survival depends on their biological properties and on the environmental conditions.

Saprophytic leptospires are naturally found in many types of wet or humid environment ranging from surface waters and moist soil to tap water. Some leptospires were even found in seawater. Both pathogenic and saprophytic species can be isolated from surface water and soils (Wynwood et al., 2014).

In warm and humid conditions, most pathogenic leptospires can survive several weeks to months in muddy soils or rivers, by mean of cellular aggregation (Trueba et al., 2004). Viable cells may persist up to 20 months after excretion and their virulence was fully preserved (Andre-Fontaine et al., 2015). Wet environments are thus an important source of leptospires, contributing to the transmission cycle of leptospirosis.

2.5. Transmission cycle

From this survival in the environment results the main transmission pathway of leptospirosis to humans: through contact with urine-contaminated environment or, less commonly, through direct contact with urine of infected animals (see Figure 4).

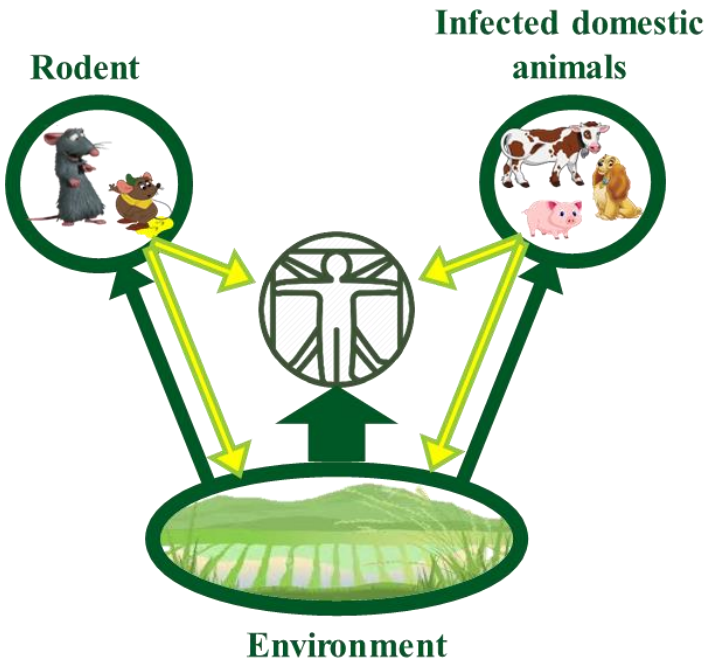

FIGURE 4 *Leptospirosis transmission cycle*
Leptospires are excreted in the urine of infected animals (yellow arrows) and can be in direct contact with humans or disposed in the environment. Most of the leptospires can survive in humid environment and will be able to infect animals or humans in contact with the contaminated environment (green arrows). This is the main transmission path of leptospirosis to human.

Leptospires may enter the body through cuts, abrasion on the skin or through the mucous membranes of the mouth, nose and eyes during swimming or (Wynwood et al., 2014). Exposures that pose a risk of transmission include splashes of infected material into the eyes, the ingestion of food or water contaminated with urine (Mwachui et al., 2015). Inhalation of water or aerosols also may result in infection via the mucous membrane of the respiratory tract. Infection may follow animal bites as it generates a skin lesion, enabling the leptospires from the urine to enter the organism. *Leptospira* may also be able to penetrate intact skin that has been in water for a long time (Levett, 2001).

There is some evidence that leptospires could be transmitted to infants through breastfeeding, causing infection. However, person-to-person transmission is rare (Koe et al., 2014 ; Bolin, Koellner, 1988).

2.6. Risk factors

Human infections may be acquired through occupational or recreational activities. The prevalence of different leptospiral serovars within a human population depend on the reservoir animals present and the serovars that they carry, as well as local environmental conditions, occupation, and agricultural practices (Bharti, 2003).

Leptospirosis is associated with activities such as livestock farming, butchering and veterinary medicine in which human are directly in contact with infected animals and their urine (Wasiński, Dutkiewicz, 2013 ; Kamath et al., 2014). For instance, assisting the delivery of a new-born from an infected animal or milking infected cows may be high risk of infection from *Leptospira interrogans* serovar hardjo and pomona (White et al., 1981).

Leptospirosis is associated as well with mining, sewer maintenance where contact with urine-contaminated environment is important (Wasiński, Dutkiewicz, 2013 ; Kamath et al., 2014).

In developed countries, many cases occur in association with recreational activities involving immersion in water kayak, swimming or adventure race (Stern et al., 2010 ; Mwachui et al., 2015).

In tropical countries where the temperature and moisture enables a longer survival of the leptospires, leptospirosis is endemic with an increase of the incidence during high seasonal rainfall and outbreaks following flooding (Dechet, 2012 ; Lau, 2010 ; Amilasan et al., 2012). In these countries where the incidence is already high, occupational exposure such as rice-farming, taro farming (Vinetz et al., 2005) and other agricultural activities increase the risk of leptospirosis (Mwachui et al., 2015).

2.7. Leptospirosis, the disease

The clinical symptoms of leptospirosis in humans varies greatly from benign forms with flu-like signs, fever, myalgia, headache and cough, to more severe forms. Weil's syndrome is characterized by jaundice, renal failure, haemorrhage and myocarditis with arrhythmias (Haake, Levett, 2015).

Disease severity varies with the infecting serovar and the dissemination of leptospires to various organs such as kidney, lung, liver, brain. This dissemination to other organs and their damage can result in hepatic, pulmonary or renal forms of leptospirosis, characterised by haemorrhages and the organ failure.

These symptoms are not pathognomonic and in endemic countries, are easily attributed to other more common diseases with similar symptoms that are distributed in the same areas, such as malaria, dengue and enteric fever (Costa et al., 2015). Such misdiagnosis lead to an underestimation of leptospirosis cases and little is actually known about the true disease burden.

Clinical diagnosis is difficult because of the varied and nonspecific presentation and undiagnosed leptospirosis can progress to more severe forms with poorer prognosis. Causes of death include renal failure, cardiopulmonary failure and widespread haemorrhage. The mortality rate varies from 5-10% for the symptomatic cases to 20-50% for the more severe forms with complications, especially in case of pulmonary haemorrhage (Haake, Levett, 2015 ; Segura, 2005).

2.8. Diagnostic tests

Numerous tests have been developed, but availability of appropriate laboratory support is still a problem. Table 1 presents a summary of the different tests and their advantages and disadvantages from Musso and Scola, (2013). Figure 5 from (Levett, 2001) is an illustration of the biphasic dynamic of leptospirosis and the relevant tests at the different stages of the disease.

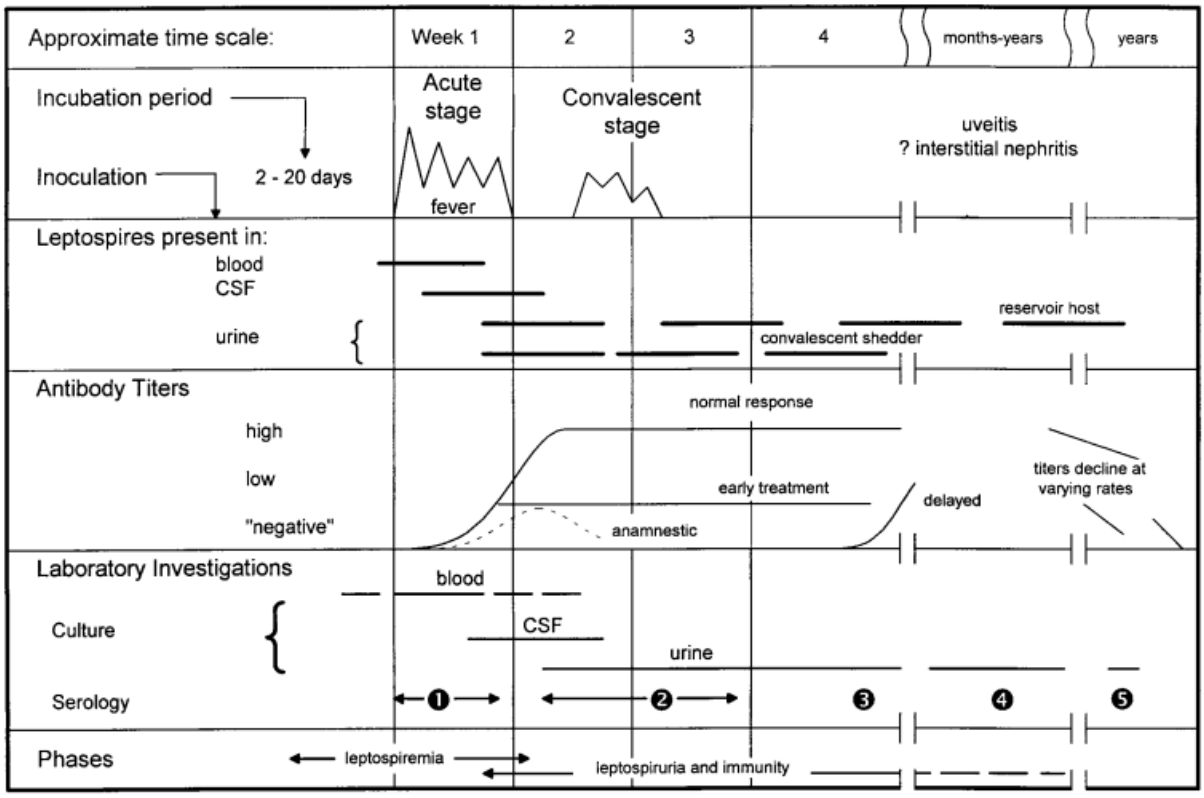

FIGURE 5 *Biphasic nature of leptospirosis and relevant investigations at different stages of disease SOURCE: LEVETT et al (2011).*

Leptospires may be visualized in clinical material by dark-field microscopy or by immunofluorescence or light microscopy after appropriate staining. Dark-field microscopy to see organisms in blood or urine is fraught with false-positives and false-negatives, is unreliable and therefore, not recommended (Musso, Scola, 2013).

Culture and isolation of leptospires from clinical samples gives a definitive diagnosis. Blood should be cultured as soon as the patient's presentation as leptospiremia occurs before the onset of symptoms and ends by the first week of the illness. Cerebrospinal fluid and dialysate fluid can also be cultured during the first week of illness and urine from the second week of symptomatic illness. The use of cultures to confirm diagnosis is rare as it is very tedious, expensive and requires prolonged incubation that can take up to months and does not contribute to early diagnosis (Musso, Scola, 2013).

Serology is the most frequently used diagnostic approach. The current gold standard is the microscopic agglutination test (MAT). Patient sera are reacted with live antigen suspensions of leptospiral serovars. After incubation, the serum-antigen mixtures are examined microscopically for agglutination and the titers are determined. This method, however, relies on the maintenance of panels of *Leptospira* serovars through culture. The MAT is complex to control and perform; it cannot be standardized because live leptospires are used as antigens (Chappel et al., 2004).

Enzyme-linked immunosorbent assay (ELISA) detects antibodies reacting with a broadly reactive genus-specific antigen and thus is not suitable for identification of the causative serovar or serogroup. Leptospiral DNA can be amplified from serum and urine. PCR detects DNA in blood in the first 5-10 days after the onset of the disease and up to the 15th day. Several primer pairs for PCR detection of leptospires have been described, some based on specific gene targets or repetitive elements. PCR is based on the detection of genes universally present in bacteria as *gyrB*, *rrs* (16S rRNA gene), *secY* or genes restricted to pathogenic *Leptospira* spp. as *lipL32*, *lfb1*, *ligA*, *ligB.* Real-time quantitative PCR, combining amplification and detection of amplified product in the same reaction vessel has an excellent sensitivity and specificity and low contamination risk. They can either be performed using SYBR Green or fluorescent TaqMan probes. Depending on the target gene, these PCR allow detection and differentiation of pathogenic and non-pathogenic leptospires from clinical and environmental samples. PCR can rapidly confirm the diagnosis in the early phase of the disease when bacteria are present and before antibody titres are at a detectable level. However, they require special equipment

and skilled technicians, lacking in some areas. Laboratory diagnosis tests are not always available, especially in developing countries.

TABLE 1 *Advantages and disadvantages of common diagnostic tests for leptospirosis*
SOURCE : Musso, Scola, (2013)

	Microscopic demonstration	Culture	Serology MAT	Serology ELISA IgM	Serology rapid tests IgM	Molecular testing
Specimen collection	Blood, urine, CSF	Blood, urine, CSF, tissues	Blood	Blood	Blood	Blood, urine, CSF, tissues
Window of positivity	1st wk: blood, CSF 2nd wk: urine	1st 10 d	From Day 10—12	From Day 6—8	From Day 6—8	From Day 5—10 in blood
Processing time	Available in 1 h	2 wk to 4 mo	Several wks if not locally available	Available in 1 d	Available in 15—30 min	Available in 1 d
Early diagnosis	No	No	No	No	No	Yes
Definitive diagnosis if positive	No	Yes	Yes (seroconversion)	Yes (seroconversion)	Yes (seroconversion)	Yes
Identification	No	Yes (if MAT or molecular testing available)	Yes	No	No	Yes (by additional molecular tests)
Remark	Low sensitivity and specificity Not recommended for diagnosis	Low sensitivity, slow, difficult	Gold standard but very difficult	Needs confirmation by MAT	Needs confirmation by MAT	The only sensitive test at the acute phase
Equipment required	Dark field microscope	Specific culture media	Reference laboratory only	Standard laboratory	Laboratory equipment not required	Special equipment, dedicated laboratory space, highly skilled personnel

2.9. Treatment

In Human, treatment of leptospirosis differs depending on the severity and duration of symptoms. Patients with mild, flu-like symptoms require only symptomatic treatment. Hospital admission is required for patients with icteric leptospirosis and dialysis for patients with acute renal failure. Severe cases of leptospirosis can be treated with high doses of intravenous penicillin. Doxycycline, ampicillin and amoxicillin are recommended in mild cases (Levett, 2001).

Vaccinations for dog and cattle are available to prevent illness but do not prevent the shedding of the bacteria and thus their transmission to human. Canine vaccines generally contain serovars canicola and icterohaemorrhagiae and can include serovars grippotyphosa and pomona (Klaasen et al., 2014). However immunized dogs may be infected with serovars other than those contained in commercial vaccines (Llewellyn et al., 2016).

Vaccines to prevent human leptospirosis are available in some countries but are not authorized in other countries. None of them protect against all circulating strains and focused only on important serovar. For instance in France, a monovalent vaccine against *L. interrogans* from Icterohaemorrhagiae serogroup is available only for sewers workers (Institut Pasteur, 2015).

Moreover, these killed bacteria vaccines are likely to provide only short-term and possibly incomplete protection (Haake, Levett, 2015).

3. LEPTOSPIROSIS EPIDEMIOLOGY

Leptospirosis is considered the most common and widespread bacterial zoonosis (Levett, 2001). The global burden of leptospirosis is estimated at around 1.03 million cases per year and 58 900 deaths per year (Costa et al., 2015). These estimates emphasize that leptospirosis is one of the greatest zoonotic causes of morbidity and mortality. Leptospirosis takes an even greater importance in tropical and subtropical regions where the disease is endemic. Over 73% of the total cases of leptospirosis worldwide are believed to occur in the tropics (Victoriano et al., 2009).

3.1. Leptospirosis epidemiology in humans, in SEA

Leptospirosis incidence in the Asia-Pacific is estimated between 10 and 100 cases per 100 000 habitants per year (Costa et al., 2015). Although transmission is endemic and large outbreaks have been reported, there is currently no routinely performed surveillance in South East Asian countries and therefore no official data on the incidence of the disease. In tropical regions, where humid and warm conditions enable a longer survival of *Leptospira*, leptospirosis is significantly associated with occupational exposure such as rice farming and other agricultural activities (Bharti, 2003 ; Mwachui et al., 2015) and with heavy rainfall or extreme weather events such as floods and cyclones (Dechet, 2012 ; Lau, 2010 ; Wasiński, Dutkiewicz, 2013). In Vietnam and Laos, leptospirosis was shown to occur in a seasonal pattern in these countries, with a peak incidence during the rainy season and outbreaks occurring after flooding (Lau, 2010).

In Cambodia, few studies give an insight into the leptospirosis situation in the country: in 2003, a survey in Takeo provincial hospital estimated the annual incidence to be 7.65 cases per 100 000 habitants, with Javania and Australis as the main serogroups (Seng et al., 2007). In 2012, fever surveillance in South-Central Cambodia found a seroprevalence of 20.8% IgM (Kasper et al., 2012). A more recent study by Institut Pasteur du Cambodge from 2007 to 2009, found a seroprevalence of 26.7% of febrile cases in patients younger than 20 years old, in Kampong Cham province. Of these, 15.8% had seroconversion illustrating the incident infection in this province. They also showed that probability of having a fever caused by leptospirosis was at 1% of all fevers per semester (Hem et al., 2016).

3.2. Leptospirosis epidemiology in rodents, in SEA

Little is known about human leptospirosis in Southeast Asia, but the lack of knowledge about leptospirosis circulation in rodents is even greater, though they are believed to be important reservoirs and could be a source of infection to humans. In a study conducted in the Mekong

Delta of Vietnam (Loan et al., 2015), 5.8% of captured rats were tested positive by RT-PCR and 18.3% by Microscopic Agglutination Test (MAT). They observed a higher prevalence of detection among older rats, suggesting a long-term carriage of leptospires and that *Leptospira* infection does not result in increased mortality in rats. In Thailand, leptospires were found in the following species: *Rattus argentiventer, R. exulans R. losea, R. norvegicus, R. tanezumi* but also in the less studied *Bandicota indica* and *B. savilei* (Herbreteau et al., 2012).

A recent study conducted in Thailand, Lao PDR and Cambodia assessed *Leptospira* prevalence in rodent species using RT-PCR, and found that detection varied from 0 to 18% across localities, and from 0 to 19% across species (figure 6, Cosson et al., 2014). In Cambodia, the prevalence was 4% in Mondolkiri province and 8.33% in Sihanoukville province and the leptospira species found, were *Leptospira borgpetersenii, L. interrogans* and *L. weilli*. *Leptospira borgpetersenii* and *L. interrogans* were the most abundant species in Lao, Thailand and Cambodia but were found in different habitats.

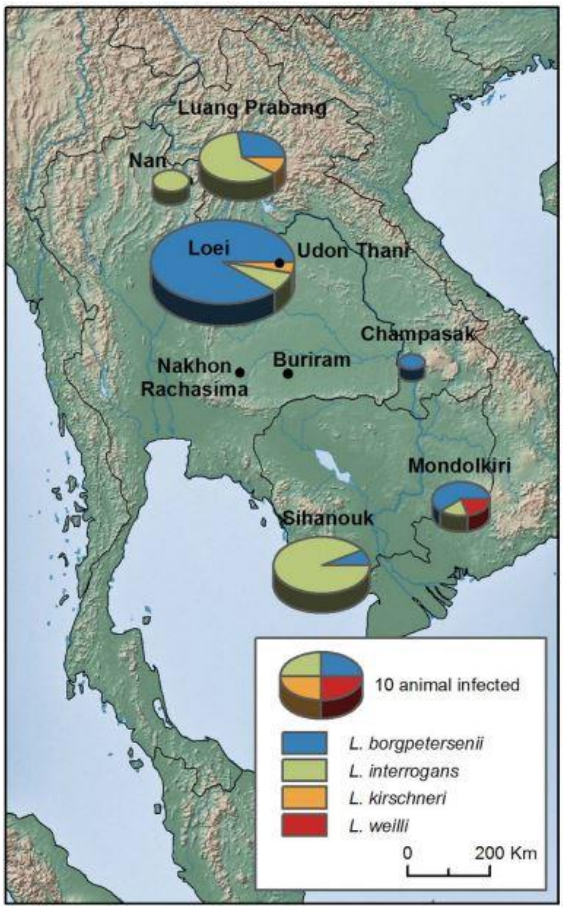

FIGURE 6 *Geographic distribution of Leptospira infection in rodents from Thailand, Lao PDR and Cambodia. SOURCE : Cosson et al (2014)*

L. borgpetersenii was more abundant in dry habitats than *L. interrogans*, suggesting a difference in ecological niche for these *Leptospira* species.

However, the study found no difference of prevalence between floodable areas, forests and dry agricultural fields. Such a result illustrates the two transmission routes of leptospirosis and suggests that direct transmission could explain the circulation of leptospires in the dry habitats while the indirect transmission via wet environments occurs in floodable areas. *Leptospira* prevalence did not significantly vary across rodent species, though higher prevalence was observed in wild mice (*Mus* sp.) and in rarely investigated forest species (*Berylmys* sp., *Maxomys* sp.).

In Cambodia, the forest species *Maxomys surifer* and *Niviventer fulvescens* were also found to carry *Leptospira* and high prevalence was observed in *Rattus argentiventer*, a species found in rain-fed cultivated areas. Leptospira were also detected in *Bandicota savilei* and *Berylmys berdmorei* which are both present in paddy fields; and *B.berdmorei* which is found in forest areas near crops. High prevalence of *Leptospira* in rodents in cultivated areas and degraded forest suggests that these habitats may present a high risk of leptospirosis for humans (Svilena Ivanova, 2012).

Rodents may move among habitats, either as part of the natural dispersal process (from their birth place to their first breeding site or from one breeding site to another) or in response to the seasonal variation in habitat quality (i.e. amount of food, shelter availability, competition with other rodents, predation etc.). In Lao PDR, movements of rodents between field and village habitat were shown in response to the availability of food resources (Douangboupha et al., 2009). Because these movements may involve rodents infected with *Leptospira*, this process could have important consequences on *Leptospira* distribution within Southeast Asian landscapes. It seems therefore likely that deforestation, by disrupting rodent habitats, would have consequences on *Leptospira* distribution. But knowledge on the impact of deforestation on pathogen circulation is currently lacking. The following paragraphs summarize the current literature on how human land-use change impacts infectious disease transmission in general and rodent-borne pathogens.

4. IMPACT OF LAND-USE CHANGE ON PATHOGENS IN SEA

South East Asia is both a hotspot of land use change and zoonotic disease outbreaks. Indeed the current increase of infectious disease emergences coincides with accelerating rates of tropical deforestation (Wilcox, Ellis, 2006). An increasing number of studies on emerging infectious diseases points to changes in land cover and land use, including forest cover change such as deforestation and forest fragmentation, as major factors contributing to the surge in infectious diseases. Some examples of pathogens whose current emergence patterns show an association with forest degradation and clearing are Ebola virus, Nipah virus, malaria and Lyme disease (Wilcox, Ellis, 2006).

4.1. Land use changes and disease prevalence

Brearley et al. (2013) reviewed the influence of human-induced landscape change on wildlife disease prevalence. Half of the papers reviewed found an increase in disease prevalence due to human-induced landscape change, while 21% identified a decrease in disease prevalence and the remainder 26% indicated that disease prevalence varied (Brearley et al., 2013). Similarly, the Gottdenker et al. review (2014) about land use change impact on infectious disease showed that more than 56.9% of reviewed studies documented increased pathogen transmission in response to anthropogenic land use change, 10.4% found decreased pathogen transmission secondary to land use change, 30% observed variable pathogen response while 2.4% showed no changes at all (Gottdenker et al., 2014). These results (illustrated in Appendix 4) clearly indicate that the issue of wildlife disease in human-modified landscape is complex and highly variable and no general trend of disease prevalence response to land-use changes has been determined. Richness of infectious diseases was found positively correlated with the richness of mammals depicting biodiversity as a source of pathogens (Morand et al., 2014). The same study also found an association between an increase of zoonotic disease outbreaks and the loss of biodiversity as measured by the number of species at threat and proportion of forest cover.

The current evidence suggests several hypothesized mechanisms that would lead to infectious disease changes after land use alteration. They include modification of habitat structure, microclimate and resource availability for both the host and its pathogens. Other proposed mechanisms were changes in the host community composition, and host jumping of the pathogen. Anthropogenically driven land use changes can produce ecological conditions that facilitate geographic expansion of pathogens via the modification of spatial distribution of the host or of their behaviour and movements (Gottdenker et al., 2014).

Deforestation and forest fragmentation can have two possible outcomes: on one hand the highly fragmented environments may reduce the disease prevalence due to lack of connectivity between the patches of forest, resulting in less contact rates between hosts and thus reducing the infection rates; conversely, such fragmentation may also increase contact rates and disease prevalence by clumping the resources and the hosts (Bradley, Altizer, 2007). Or, in case of indirectly transmitted diseases such as leptospirosis, fragmentation may result in overlapping distributions of species and might increase their probability of getting infected from each other, from the spread of germs along the tracks they now share. Furthermore, deforestation leads to ecological changes such as increased edge habitat and local extinction of predators that may favour some species that happen to be disease reservoir (Wilcox, Ellis, 2006).

4.2. Land use changes and rodent-borne diseases

The conversion and loss of forest in SEA is presumed to affect rodents in terms of their diversity and species composition. Several studies have established that rodent species may differ in their response (presence/absence or abundance) to habitat modification. The importance of habitat modification in shaping small mammal communities is now recognized and was assessed by world-wide studies (Bernard et al., 2009 ; Morand et al., 2015 ; Lynam, Billick, 1999 ; Umetsu, Pardini, 2007). Bernard et al. (2009) found that habitat types (forest versus plantation) were important determinant of small mammals' species occurrences and assemblage composition in Borneo.

Morand et al. (2015) showed that habitat structure and fragmentation affected the spatial distribution of rodent species in SEA. In particular, alteration of the habitat (decreasing forest cover, increasing fragmentation and urbanization) was found to favour the presence of synanthropic rodent species such as *Rattus tanezumi*. Similarly, Suzán et al. (2008) highlighted that fragmentation of the habitats resulted in lower diversity of small mammals and higher densities of populations of rodents. All these studies focusing on forest fragmentation in tropical areas have emphasized that species richness or diversity are affected in disturbed ecosystems.

Changes in land use in Southeast Asia are therefore expected to alter rodent species distribution and diversity (Morand et al., 2015). As rodents are important reservoirs of human diseases, it is likely that their incidence is linked to fluctuations in rodent population. Habitat fragmentation and decreasing forest cover seemed to favour the presence of synanthropic rodent species that are important host of rodent-borne diseases (Suzán, Marcé, et al., 2008). In particular,

Bartonella spp. and hantavirus were associated with disturbed landscapes with ongoing fragmentation (Morand et al., 2015).

Outbreaks of hantavirus diseases, were found to occur in anthropologically disturbed habitats, where natural biodiversity had been reduced to low levels (Mills, 2006). This was explained by the fact that disturbed habitats favour opportunistic species that were reservoir of hantavirus and due to the lack of competitive pressure, these species could reach higher densities. It is therefore important to understand which rodent-borne pathogens would have a similar response to land use changes as those observed for hantavirus.

4.3. Land-use changes and leptospirosis

Studies conducted in SEA have found leptospires in all types of rodent habitats: from forest to floodable areas and dry agricultural fields (Cosson et al., 2014 ; Ivanova et al., 2012).

Ivanova et al. (2012) described a high prevalence in rodents trapped in newly cultivated areas and in degraded forests and that species from these two areas had similar level of infection. This study suggested that both rice fields and forests were also areas of potential risk for leptospirosis. Though it is important to note that *Leptospira* was detected in this study with the PCR protocol from Mérien et al (1992) that detects both pathogenic and non-pathogenic species.

Morand et al (2015) observed associations between habitat structure (forest, settlement, agricultural fields and fragmented habitats) and the prevalence of rodent-borne pathogens with *Leptospira* prevalence linked to fragmented habitat (Figure 7). This discovery highlighted fragmented forest as a zone of higher risk of leptospirosis than in paddy fields.

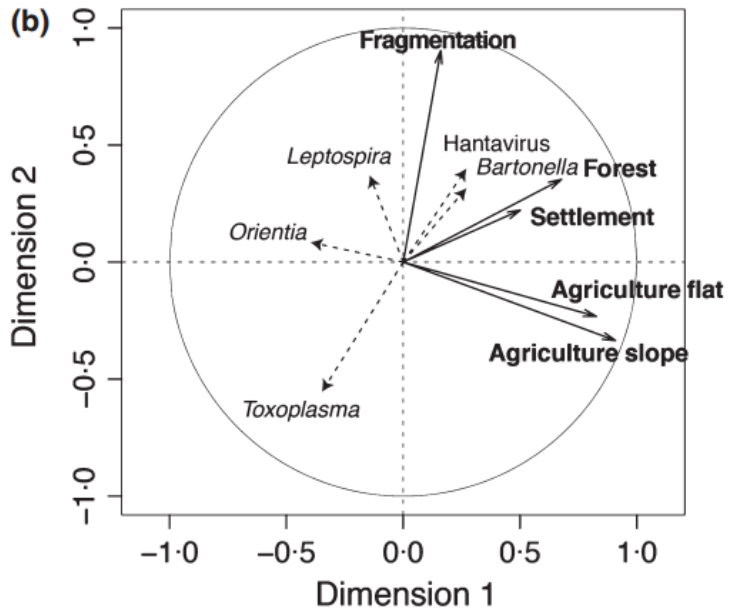

FIGURE 7 *Associations between habitat structure and the prevalence of rodent-borne pathogens, based on values on the first two axes of the principal component analysis from Morand's study (2015).*
SOURCE : Morand et al 2015

Leptospirosis has been linked with weather-related phenomena such as flooding but also with anthropogenic land-use changes that influence the natural flow of water (Vinetz et al., 2005). Indeed, they advanced that hydrologic alterations of watersheds may impact the survival of *Leptospira* in the environment and could consequently influence the disease incidence. Even though these previously cited studies have stressed that anthropological changes may increase leptospirosis risk, there is no evidence yet of a direct causal relationship between deforestation and leptospirosis. Respective limits of the studies do not permit us to clear establishment of a link between them and are detailed in the following section.

4.4. Limitations of the current literature

The literature on land use changes and infectious disease, although a field of growing concern, is quite limited. A third of the published studies are actually review papers and almost all of the original research papers are observational studies (Gottdenker et al., 2014). There is a lack of experimental or analytical studies evaluating the relationship between environmental changes and infectious disease transmission (Gottdenker et al., 2014). The majority of past studies were observational approaches based on a one-dimensional comparison of prevalence or vector abundance at landscape level (between unmodified and modified sites) and presumably had a major influence on the variability of results. Studies conducted at a single spatial scale can explain only partially the impact of human-modified heterogeneous landscapes (Gottdenker et al., 2014). Indeed characteristics present at a landscape-scale may have different impacts on

wildlife disease compared to characteristics present at habitat or micro-habitat scales. Studies conducted at multiple spatial scales will provide a more complete understanding of the processes influencing the species distribution, abundance and leptospirosis prevalence according to Brearley et al. (2013). They also indicated that none of the studies linked temporal changes in landscape modification with consequences in wildlife disease prevalence and therefore recommended that future research should incorporate temporal components as well as spatial components of analysis. Indeed, it stands to reason that land use change is inherently a temporal process and that disease prevalence is likely to also vary over time. Studies comparing the disease prevalence before and after deforestation will not depict the actual risk of disease and will overlook out the changes of disease prevalence that may occur during the deforestation process.

During land-use change there may be incremental alterations in the environment from the virgin forest to the long-term plantation field. These may include selective logging and forest degradation, increase forest patchiness (fragmentation), secondary growth of undergrowth, invasive plant species and secondary forest, burning, and immature grassland before the long-term steady-state situation of land cultivation. Bradley termed this transient period "chronotone" and defined it as "*a boundary in time between two ecosystems or landscape phases, by analogy with "ecotone" that is the boundary in space between two ecosystems.*" (Bradley, 2004).

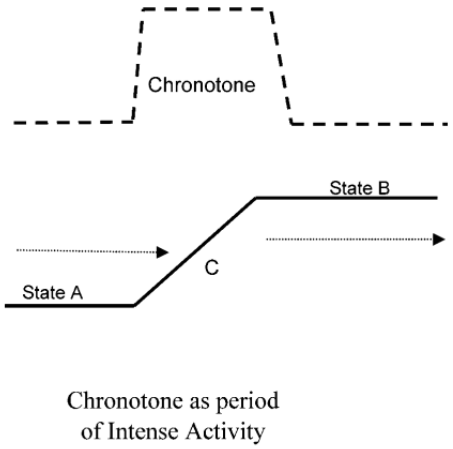

FIGURE 8 *Chronotone representation as a period of intense activity (C). The chronotone is the transient period of environmental change but also in time of the ecosystem from one state (A) to another (B).*
SOURCE : Bradley, 2004

This transition phase (e.g. between forest and agricultural land) is of great importance in the epidemiology of infectious diseases in changing landscapes as it includes a succession of mechanisms affecting habitats, hosts communities and diseases dynamics in the unstable ecosystems. Study the deforestation chronotone is thus likely to provide important insights into the impact of deforestation on leptospirosis prevalence in rodents, and the associated risk to public health.

PART 3: HYPOTHESIS

PART 3: HYPOTHESIS

The main objective of our study is to assess the consequences of the changes occurring during the process of deforestation on rodent populations and on the dynamics of leptospirosis.

Our aims are:

- To demonstrate the shift of rodent species composition and the ecological mixing of different rodent species accompanying rapid deforestation
- To determine the species carrying *Leptospira*.
- To identify the *Leptospira* strains carried by the different rodents in the different steps of deforestation to assess the potential risk for human health.

Studying the changes driven by deforestation on rodent species distribution, densities and prevalence would allow an assessment of the possible risk of human leptospirosis. Our hypothesis is that the transition period from intact forest to cleared forest, represents an opportunity for an increased circulation of *Leptospira* spp between rodent species. We predicted that the ongoing changes, driven by deforestation, affect the distribution of rodents and thus of the pathogens they carry.

Specific hypothesis:

- The disruption of the habitat modifies the distribution of rodent species and their densities, resulting in overlapping distributions of different species that were originally not in contact.
- Deforestation increases the risk of leptospirosis:
  - By transmission of *Leptospira* from a wild species from the intact forest to other rodent species that are invading the disrupted forest.
  - By introduction of *Leptospira* by invasive species, that are non-native to the forest and whose introduction and spread are likely to cause harm to human health.
  - By favouring species which are *Leptospira* hosts.

PART 4: METHOD AND MATERIALS

PART 4: METHOD AND MATERIALS

1. Study design

To study the deforestation chronotone, i.e. the succession of ecological and epidemiological changes, we used a chronosequence design that compares areas representative of different steps of the deforestation process. This method involves a space-for-time substitution that assumes that the different zones that are compared only differ by their time since the beginning of the deforestation process, and can be considered a time sequence. Three levels of forest degradation were defined as follow:

INTACT FORST: intact evergreen or semi-evergreen forest protected areas or community forestry, with low to medium selective logging (**Zone 1**);

DISTURBED FOREST: degraded forest with intensive tree removal (**Zone 2**);

RECENT FIELD: recent plantation less than one year since clearing (**Zone 3**).

These zones corresponded to different steps of forest clearing and thus to different steps of the deforestation chronotone. The main assumptions of this design is that the three zones had the same initial stage and that they follow the same pattern of change. To ensure these assumptions were met, the three zones (corresponding to the three steps of forest degradation) were matched within each site included in this study. This matching was designed to minimize the spatio-temporal variations that would be unrelated to the process of deforestation and thus ensured that zones were comparable. All matched zones of a site were sampled at the same time. In addition, the three zones of each site were sampled in two seasons: rainy season from June to September, and dry season from January to April.

1.1. Trapping grid

Fifty locally made non-lethal Havahart traps were deployed in each of the three zones, giving 150 traps per site. The traps were separated by 20-meter intervals and placed at least 100m from the habitat edge in each zone. Zones were at least 200m apart from each other. Traps were deployed during eight consecutive nights and were baited with sweet-potato covered with peanut butter. The sampling effort was comprised by a total of 1200 night traps per trapping. Each trap was located with a global positioning system (GPS) receiver and the surrounding habitat was characterized by its canopy cover, vegetation density and vegetation transects around the traps (data not shown). The geography of each site and an example of a grid layout from one site are provided in the appendix 5 and 6.

Trapping effort was identical between zones and capture session occurred simultaneously in the zones of a same site. This capture design allowed to minimize the effects of temporal variation and weather on capture probability.

1.2. Rodent sampling

Captured rodents were anaesthetised for a short period after inhalation of isoflurane and then sampled in a modified field biosafety container to ensure the safety of field staff. Blood, feces or rectal swab, urine or uro-genital swab, oral swab, skin and ectoparasites (fleas, ticks and chiggers) were collected from each animal and preserved in both viral transport media and RNAlater (except ectoparasites which were preserved in 70% ethanol). Rodent identifications (species, age, sex, etc.) were recorded (detailed in Appendix 7) and each rodent was marked with a unique ear tag and released at its original capture location. Recapture was recorded with the ear tag of the recaptured animal and the trap number. This capture-mark-recapture design enabled the assessment of the overall recapture rate, population density and home range size.

2. Laboratory analyses

2.1. Rodent species

Rodent species were identified on the basis of their morphology (Francis et al., 2008) and by barcoding assignment. Skin tissue was collected under anaesthesia, from a 1 mm ear punch or the tip of the tail and DNA was extracted from the tissue using the Qiagen DNeasy® Blood & Tissue Kit according to the manufacturer's instructions. The primer set of BatL5310 (CCTACTCRGCCATTTTACCTATG) and R6036R (ACTTCTGGGTGTCCAAAGAATCA) were used to amplify a 750 base pair fragment of the Cytochrome c oxydase I (COI) gene, as previously used in the CERo-Path project (Pagès et al., 2010 ; Svilena Ivanova, 2012). PCR products were visualised by gel electrophoresis before amplicons were sent for sequencing to Macrogen (Seoul, South Korea). Sequences were trimmed and assembled using CLC Genomics Workbench 3.6.1 (2013) software. Either the consensus (when obtained) or both sequences (reverse and forward) were submitted for BLASTn search to obtain the species on the NCBI website (National Center for Biotechnology Information) or CERo-Path website (CERoPath, http://www.ceropath.org/). An exemple is given on Appendix 8.

2.2. *Leptospira* detection

Urine, uro-genital swabs, rectal swabs and feces were used to identify rodent carriers of *Leptospira*. DNA was extracted using the Qiagen RNeasy® Mini Kit. We performed two

different PCRs to detect *Leptospira.* The first PCR amplified the rrs-gene, universally present in *Leptospira* and thus detected both pathogenic and intermediate pathogenic *Leptospira* species allowing us to carry out a broader screening of *Leptospira* in rodents (*Leptospira* species assay). The second PCR amplified the LipL32 gene, which is only present in pathogenic *Leptospira* spp. and encodes an outer membrane lipoprotein that is considered to be a virulence factor (Haake, 2000). This second PCR permitted us to detect species of *Leptospira* that are known to be human pathogens (Pathogenic *Leptospira* assay).

2.2.1. *Leptospira* species assay

This real-time PCR assay, previously described by Thaidunpanit, Slack and Smith, amplified the rrs-gene. The primer set of Lepto-F (5'-CCCGCGTCCGATTAG-3') and Lepto-R (5'-TCCATTGTGGCCGRACAC-3') were used to amplify the rrs-gene detected by the probe Lepto-probe (5'-6-FAM-CTCACCAAGGCGACGATCGGTAGC-BHQ1-3'). Real-time amplification was performed using the BioRad Thermal Cycler CFX96. The PCR reaction conditions were as follow: 4.25 mmol/L of MgCl2, 0.25 µmol/L for the Primer F (Lepto-F), 0.50µmol/L for the Primer R (Lepto-R), 0.05 µmol/Lf of Lepto-probe and 5µL of DNA extract in a final volume of 20µL. PCR amplification was performed using the following parameters: reverse transcription for 2 min at 50°C thenan initial denaturation at 95°C for 8 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing elongation at 60°C for 1 min. Positive samples were defined as having a cycle-to-threshold (CT) value below 35.

2.2.2. Pathogenic *Leptospira* assay

Detection of human pathogenic *Leptospira* infection was determined by real-time PCR, using a TaqMan assay targeting the LipL32 gene. The primer set of LipL32-45F (5′-AAG CAT TAC CGC TTG TGG TG-3′) and LipL32-286R (5′-GAA CTC CCA TTT CAG CGA TT-3′) were selected to amplify a fragment of 242 bp, which was detected by the probe, LipL32-189P (FAM-5′-AA AGC CAG GAC AAG CGC CG-3′-BHQ1), as previously reported (Stoddard et al., 2009). Reaction conditions used were 3.0 mmol/L of MgCl2, 400 nmol/L of each primer, 132.5 nmol/L probe, and 5µL of DNA extract in a final volume of 20µL. The amplification protocol consisted of 3 min at 50°C then pre-denaturation at 95°C for 8 min, followed by 45 cycles of amplification (95 °C for 20 s and 58 °C for 40 s). Samples with a CT value below 40 were considered positive.

2.3. *Leptospira* species identification

The protocol used was modified from a single tube nested PCR, developed by Mahidol-Oxford Tropical Medicine Research Unit (Boonsilp et al., 2011). It was used to amplify a 443-nucleotide fragment from the rrs gene.

Primers were as follows: rrs-outer-F (5'-CTCAGAACTAACGCTGGCGGCGCG-3'), rrs-outer-R (5'-GGTTCGTTACTGAGGGTTAAAACCCCC-3'), rrs-inner-F (5'-CTGGCGGCGCGTCTTA-3'), and rrs-inner-R (5'-GTTTTCACACCTGACTTACA-3').

The resulting amplicon was 547 bp. Amplicons were visualized using a 1.5% gel electrophoresis followed by staining with ethidium bromide. Samples with an amplicon of the expected size were sequenced by the Institut Pasteur du Cambodge using a combination of all four primers to generate a contiguous sequence. *Leptospira* species were identified by BLAST searches of the resulting sequences, using the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). Sequences were trimmed and aligned with CLC Genomic workbench. The phylogenetic tree was constructed from the resulting 481-bp rrs fragments alignment and a bootstrapped test with 100 replicates was performed using the (BIONJ) Neighbour-Joining method in Seaview software version 4.

3. Statistical Analyses

3.1. Rodent species richness and diversity indices

We first described the zones with their rodent species richness, diversity and their total rodent density.

Rodent species richness and diversity indices were assessed for each zone within all sites: species richness was defined as the number of species present within a zone and species diversity was calculated using the Shannon index defined by Hill (Shannon-Weaver index: $H= -\sum_{i=1}^{S} p_i log_b(p_i)$, where *pi* is the proportion of species *i* and *S* the number of species in the community—Hill 1973) vegan R package. Species richness gave us a straightforward count of identified species in the different rodent communities along the three stages of deforestation and species diversity determined the weighted presence of each species within the community.

3.2. Rodent density

Rodent densities were estimated with the null spatially explicit model in 'secr' R package using the recapture history and the GPS coordinates of capture locations, for each zone of each site. Rodent densities were then log-transformed to meet assumptions of normality.

A split-plot factorial ANOVA was used to test for differences of richness, diversity and density between zones. Site represented grouping in time and in space of the three zones. We were interested in the main effects of zones on species richness, diversity and density, and in the interaction between zones and the season. As we were not interested in testing hypotheses about differences between sites, site was defined as a random effect in our ANOVA and Season and Zone as fixed effects. Statistical significance was set at $P< 0.05$ and the Bonferroni procedure was used to adjust the P values in pairwise comparisons. Before analysis, the homogeneity of variances among zones was tested for the two indices using the Bartlett test and normality of the residuals was tested using the Shapiro-Wilk test.

3.3. Rodent species composition

To quantify the compositional dissimilarity between zones, we generated Bray-Curtis dissimilarity on the rodent species abundance matrix by zone per site, for the rainy and dry seasons separately, with the 'vegan' package in R (Bray-Curtis dissimilarity:$BC_{ij} = 1 - \frac{2C_{ij}}{S_i+S_j}$, where C_{ij} is the sum of the lesser values for only the species in common between two zones, S_i and S_j are the total number of specimens counted at both zones).

To investigate whether deforestation impacts species assemblages, we compared zones within each site for both seasons with a non-parametric analysis of variance (ANOVA) with the Adonis function in the 'vegan' package in R. Pairwise-comparisons were then to identify which zones shared similar species composition and which ones were clearly dissimilar.

Then, to illustrate the distribution of rodent species across zones, we performed a correspondence analysis (COA) on the number of individual rodents trapped in each zone per site, for the rainy and the dry season separately. We completed the COA with between-class analysis to discriminate the zones, given the distribution of species using the package 'ade 4' implemented in R. Permutation test (Monte-Carlo test) was used to assess the statistical significance of the between class analyses. This analysis allowed us to identify the species present in the different zones and their apparent association with the zones.

3.4. Habitat specialization and Zone Preference

To clearly establish the degree of habitat specialization of each rodent species regarding the three zones, we first used the previously described Shannon index, measuring the diversity of habitat (zones) for each species with the vegan R package. For species with high diversity of zones, i.e. that were not specialised for one zone and were captured in two or three zones, we modelled their zone preference as explained next. The zone preference of each species was

modelled with a nested negative binomial regression of the number of individuals for each species, with zone and season as fixed effects and site as a grouping factor, using the xtnbreg function in STATA 14.1. We also tested the existence of interaction between zone and season for species in the model to assess the seasonality of their zone preference.

3.5. *Leptospira* infection

General *Leptospira* prevalence was measured for all species in each zone of each site from the results of the *Leptospira*-assay. The χ^2 test was used to test the frequency distribution of *Leptospira* in rodent species, zones and seasons. We analysed the probability of presence/absence of general *Leptospira* infection (*Leptospira* species essay) as follows:

To test the effect of zone and season on the risk of general *Leptospira* infection, we used a conditional logistic regression with site as a grouping variable using clogit function in STATA.

To test the association with individual rodent characteristics: species, age (juvenile, adult) and sex on the risk of infection, we used a conditional logistic regression as well, using clogit function in STATA. Trapping session of the five sites occurred at different time during each season and can therefore, induce potential selection bias as capture probability was quite probably different given a different time and different weather. Grouping on site enabled to minimize this bias and compare only zones within the same site.

Cases were individual rodents. Statistical significance was set at $P< 0.05$.

PART 5: RESULTS

PART 5: RESULTS

1. Rodent trapping

Rodents were trapped in the Cambodian provinces of Mondulkiri, in the Keo Siema district for the sites S1, S2 and S4 and of Kampong Thom, San Dan district for the sites S3 and S5. A map is given in Appendix1.

A total of 547 animals were captured from the rainy and dry seasons (424 and 123 animals, respectively) (TABLE 2). Seven animals escaped before sampling and species could not be determined for another four individuals. Fourteen animals belonged to the Tupaiidae family (treeshrew) and were not included in this analysis. Rodent genotyping was successfully determined for all 522 remaining individual rodents using COI-gene barcoding and identified eleven species.

TABLE 2 *Species identity and total number of captured individuals by zone and by season. Species are ordered according to their total abundance. (Zone1= Intact forest ; Zone2=Disturbed forest ; Zone3= Recently cleared fields).*

Species	Rainy Season 2015			Total Rainy	Dry Season 2016			Total Dry	**Total**
	Zone1	Zone2	Zone3		Zone1	Zone2	Zone3		
Mus cervicolor	0	39	228	**267**	0	36	33	**69**	**336**
Rattus sp. R3	3	42	21	**66**	5	13	1	**19**	**85**
Maxomys surifer	34	15	0	**49**	17	9	0	**26**	**75**
Mus caroli	0	0	6	**6**	0	0	0	**0**	**6**
Niviventer fulvescens	0	4	0	**4**	1	1	0	**2**	**6**
Rattus exulans	0	0	4	**4**	0	0	0	**0**	**4**
Berylmys berdmorei	1	1	0	**2**	0	1	0	**1**	**3**
Vanderleuria oleracea	0	0	0	**0**	0	2	1	**3**	**3**
Berylmys bowersi	0	0	0	**0**	2	0	0	**2**	**2**
Leopoldamys sabanus	0	0	0	**0**	1	0	0	**1**	**1**
Rattus andamanensis	1	0	0	**1**	0	0	0	**0**	**1**
Total	**39**	**101**	**259**	**399**	**26**	**62**	**35**	**123**	**522**
Non Rodentia sp									
*Tupaia belangeri**	2	4	0	**6**	1	7	0	**8**	**14**

* Scandentia order, Tupaiidae family

In the rainy season, 399 rodents came from eight species and in dry season, 123 from eight species.

Mus cervicolor constituted the largest number of captured animals (64.4% of all captured animals) during both seasons. The second most abundant species was *Rattus sp.R3* (16.3%), followed by *Maxomys surifer* (14.4%). These three species accounted for 95% of the overall rodent community. Each of the other 8 species were represented by no more than 6 specimens. The detailed number of captured individuals by season, site, zone and species are presented in Appendix 9.

2. Ecological changes during deforestation

2.1. Species richness

The species richness of each zone varied from 1 to 5 species with higher species richness observed in disturbed forest (Figure 9) and was statistically different among zones (Split plot factorial ANOVA, F(2,16)=10.4, P=0.0013). The impact of zone on species richness was influenced by the season (significant interaction in the split plot factorial ANOVA F(2,16)=5.6, P=0.014). Comparisons with contrasts showed that the mean richness was significantly higher in disturbed forest than in intact forest (F(1,16)=15.6, Bonferoni-adjusted P=0.002) and higher than in recently cleared fields (F(1,16)=5.2, Bonferoni-adjusted P=0.003) The mean richness was higher in disturbed forest than intact forest regardless of the season. In contrast, the difference of species mean richness between disturbed forest and recently cleared fields was influenced by the seasons (F(1,17)=11.1, P=0.004). Recently cleared fields had a lower mean richness than disturbed forest during the dry season only.

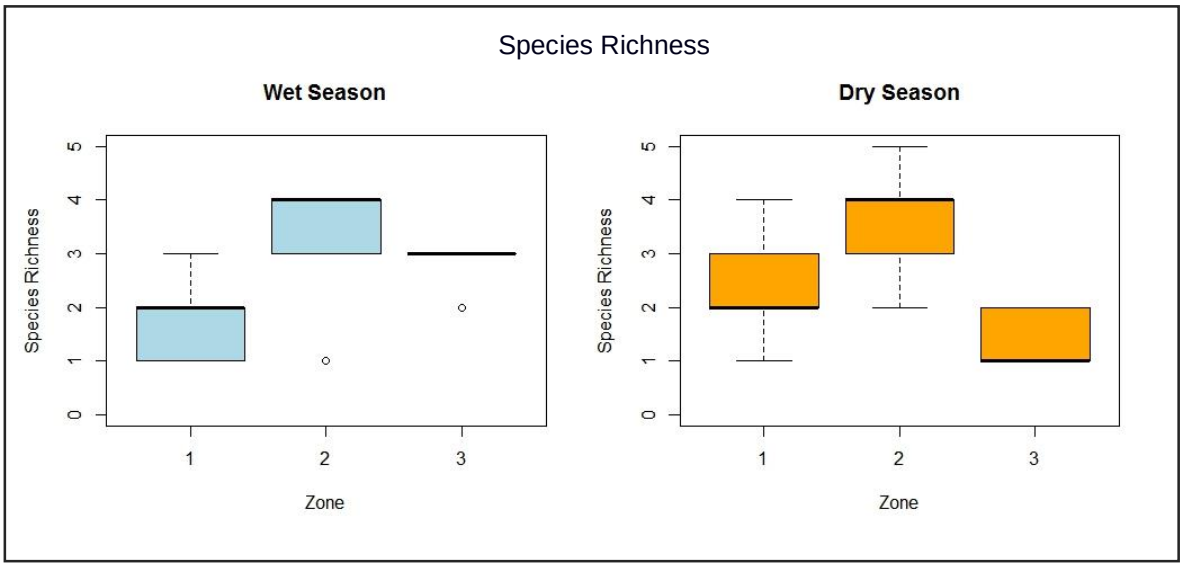

FIGURE 9 *Species richness distribution among zones by season.*
(Zone1=intact forest, Zone2=disturbed forest, Zone3=recently cleared field).

2.2. Species diversity

Species diversity varied from 0 to 1.2 (Figure 10). A zero value of the Shannon Index illustrated an absence of rodent species diversity in zones that were represented by a single species. Higher values of the index represented higher diversity.

Species diversity was statistically different among zones within sites (Split plot factorial ANOVA F(2,16)=10.9, P=0.001) and the season did not significantly influence the general effect of the zone on the species diversity (F(2,16)=2.8, p=0.08). Average diversity significantly increased from intact forest to disturbed forest (F(1,16)=7.5, Bonferoni-adjusted P=0.02) and the interaction with season was not significant. There was a significant drop of the mean diversity between disturbed forest and recently cleared fields (F(1,16)=14.2, Bonferoni-adjusted P=0.003). Thus disturbed forests were found to have the highest species richness and diversity compared to intact forest and recently cleared fields.

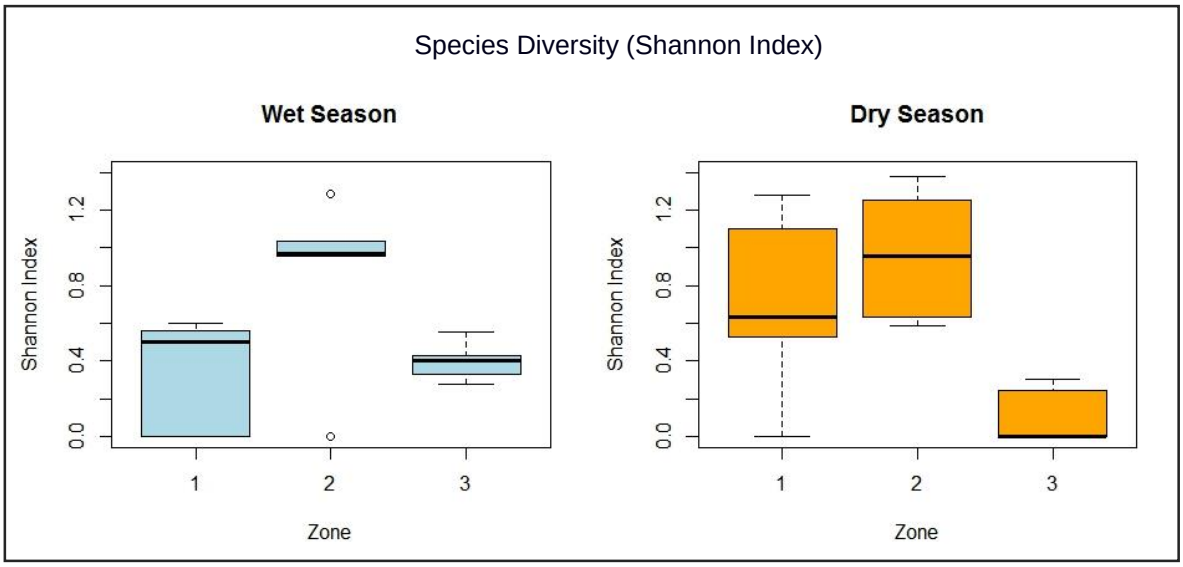

FIGURE 10 *Species diversity (Shannon Index) among zones and season (Zone1=intact forest, Zone2=disturbed forest, Zone3=recently cleared field).*

2.3. Rodent density

The total rodent density varied from 0 to 48 animals/ha. We observed clear differences between zones during the rainy season with an increase of density along the deforestation gradient. Intact forest and recently cleared fields had the highest densities and the greatest variation of density among sites. Density in intact forest and disturbed forest did not seem to vary between the seasons but we observed a sharp fall of densities during the dry season in the recently cleared fields.

Rodent density estimates (after Log+1-transformation) varied significantly among zones (Split plot factorial ANOVA $F(2,16)=10.6$, $P=0.001$) and the zone effect on density was influenced by the season ($F(2,16)=5.8$, $P=0.013$). Disturbed forest had a significantly higher mean density than intact forest ($F(1,16)=6.1$, Bonferoni-adjusted $P=0.049$) and significant lower than recently cleared fields ($F(1,16)=15$, Bonferoni-adjusted $P=0.002$). The season did not change the trend of difference between intact forest and disturbed forest but significantly influenced the difference between disturbed forest and recently cleared fields ($F(1,16)=11.4$, $P=0.004$), with decreasing densities from disturbed forest to recently cleared fields in dry season (Figure 11).

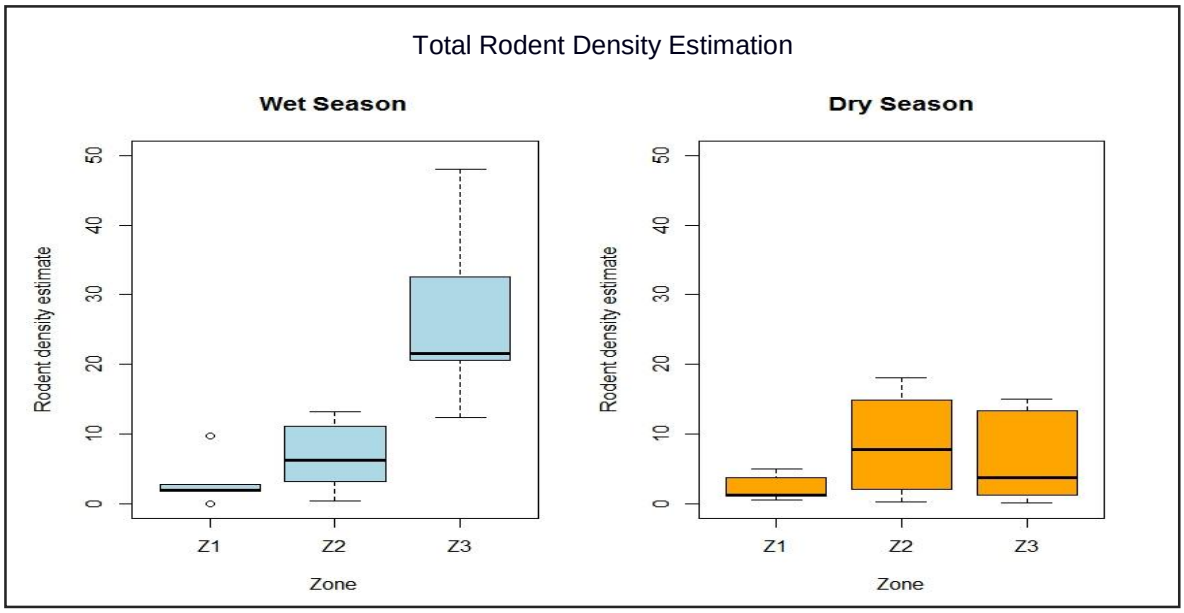

FIGURE 11*Rodent density estimate (in animal/ha) per zone by season*
Z1=intact forest, Z2=disturbed forest, Z3=recently cleared field.

2.4. Species composition

Analysis of the Bray-Curtis dissimilarity matrix between zones and sites showed that rodent species composition was statistically different among zones during both the wet season (PERMANOVA, $F(2,12)=6.8$, $P=0.002$) and the dry season (PERMANOVA, $F(2,12)=4.6$, $P=0.001$). Intact forest and recently cleared fields had the greatest dissimilarity during both seasons (PERMANOVA, $F(1,12)=7.1$, Bonferoni-adjusted $P=0.004$ for the wet season and $F(1,12)=9$, Bonferoni-adjusted $P=0.002$ for the dry season). Based on this index, there was no statistically significant difference in rodent composition between intact forest and disturbed forest. The difference of rodent species composition between disturbed forest and recently cleared fields was season-dependent. Indeed, we found a significant dissimilarity between them during the rainy season (PERMANOVA, $F(1,12)=5.9$, Bonferoni-adjusted $P=0.026$) but this dissimilarity was no longer found during the dry season. Disturbed forest and recently cleared fields therefore appeared to be more similar in their rodent species composition during the dry season than during the wet season. Because we found clear differences between zones, it is therefore of interest to look more closely on how deforestation impacts rodent species composition. In particular trying to understand changes in the presence and abundance of species during the deforestation process and to determine which of these species are *Leptospira* carriers.

The correspondence analysis plot (figures 12 & 13) illustrates the distribution of rodent species across the zones of each sites per season. The first and second axes explained 65.5% of the total inertia for the rainy season and 65.3% for the dry season. Three axes were kept for the dry seasons and they explained 82.23% of the total inertia.

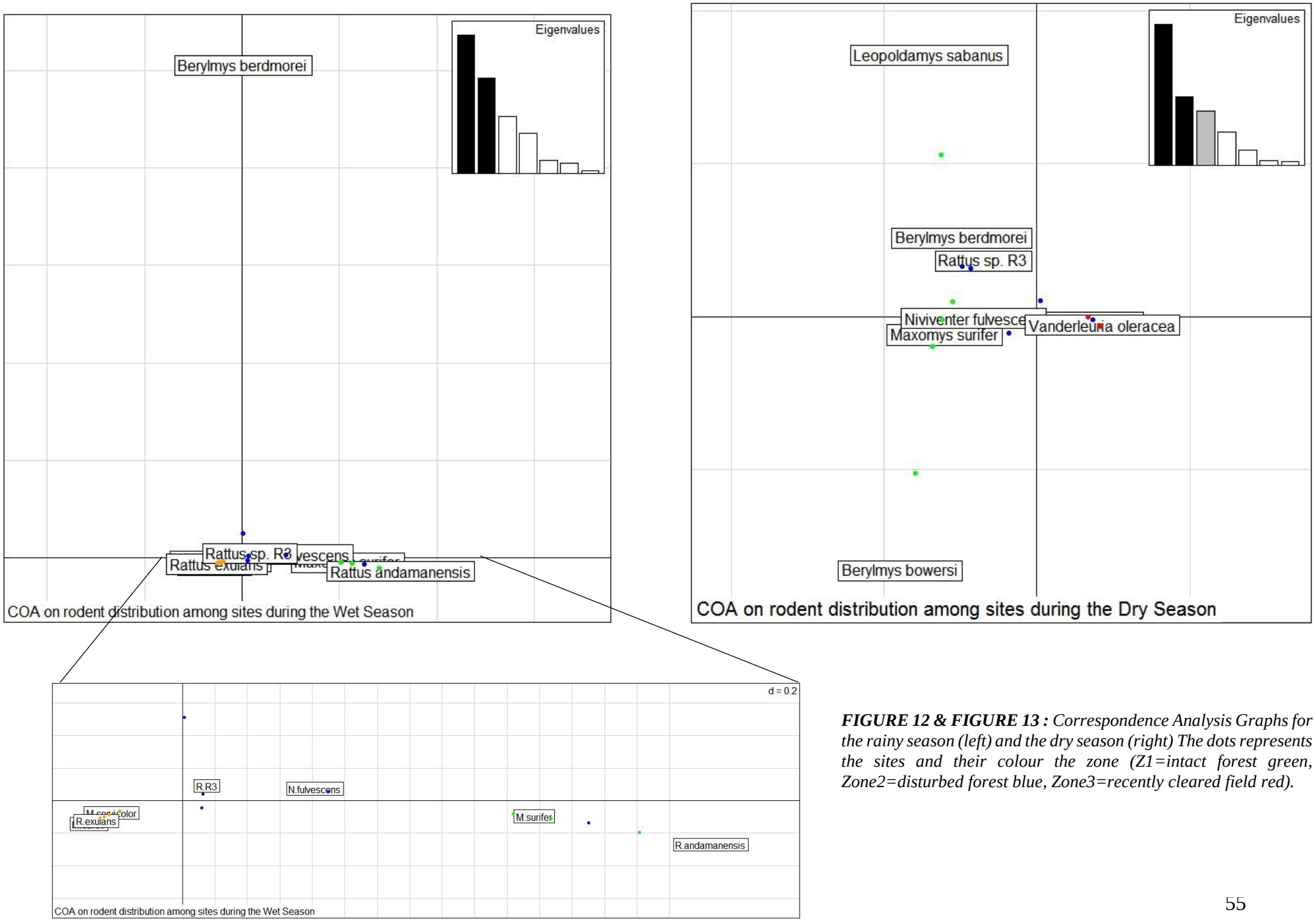

FIGURE 12 & FIGURE 13 : *Correspondence Analysis Graphs for the rainy season (left) and the dry season (right) The dots represents the sites and their colour the zone (Z1=intact forest green, Zone2=disturbed forest blue, Zone3=recently cleared field red).*

The figures 14 & 15 represent the distribution of rodent species according to sites and zones on the first two axes of a correspondence analysis (COA) for the rainy and the dry season respectively.

In the rainy season, sites and species from intact forest and recently cleared fields are clearly segregated along the first axis. The second axis is strongly associated with *B. berdmorei.* All sites lined up on the first axis which could be the results from the spatio-temporal gradient. *Maxomys surifer* and *Rattus andamanensis* demonstrated relatively strong association with intact forest. *Mus cervicolor, Mus caroli* and *Rattus exulans* tended to prefer recently cleared fields but the preference was not strong on the first axis. Other species showed lower habitat preferences such as *Niviventer fulvescens* which were found in disturbed forest and intact forest and finally *Rattus sp.R3* demonstrated a more generalist trend and was trapped in all three zones.

The between-class analysis plot confirmed these zone preferences by regrouping species and sites by zone. The zone class was significant in the rainy and dry seasons (Monte-Carlo permutation test, based on 999 replicates, p=0.001 and p=0.022, respectively). *Maxomys surifer* was present in both intact forest and disturbed forest, but was more abundant in intact forest, represented by the position of this species at the junction of intact forest and disturbed forest ellipses and closest to intact forest group. *Rattus sp.R3* seemed strongly associated to disturbed forest and *Mus cervicolor* to recently cleared fields. The greater species diversity of disturbed forest was visible with the long arrows around its gravity centre (sites less homogenous within that group) with species from intact forest and recently cleared fields, disturbed forest, in line with previous findings.

Disturbed forest and intact forest shared more species than with recently cleared fields and had more similar species compositions during the rainy season. This trend was reversed during the dry season, where all species captured in recently cleared fields were also present in disturbed forest (illustrated by closer ellipses and overlapping species in figure 14).

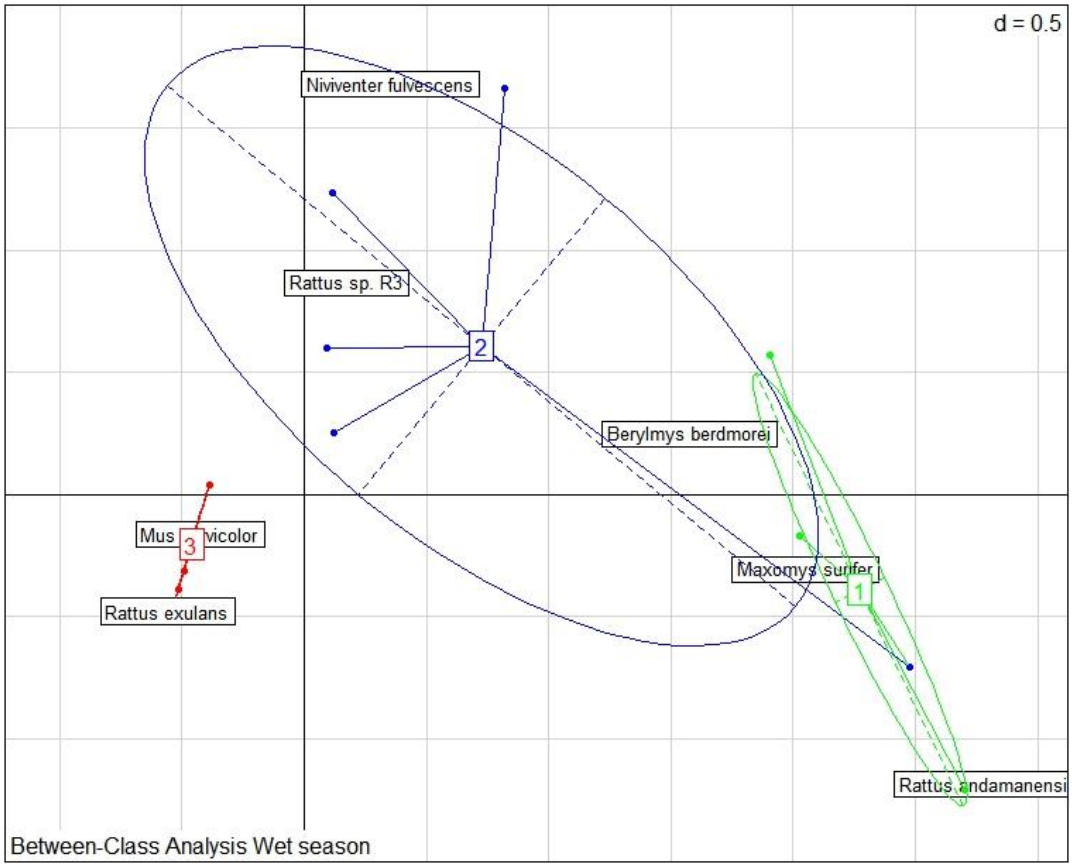

FIGURE 14 *Between-class correspondence analysis of species abundance among the three zones. The dots represent the sites, and colors the zones (Z1=intact forest green, Zone2=disturbed forest blue, Zone3=recently cleared field red). The sites are linked to the class-gravity, center of the ellipse.*

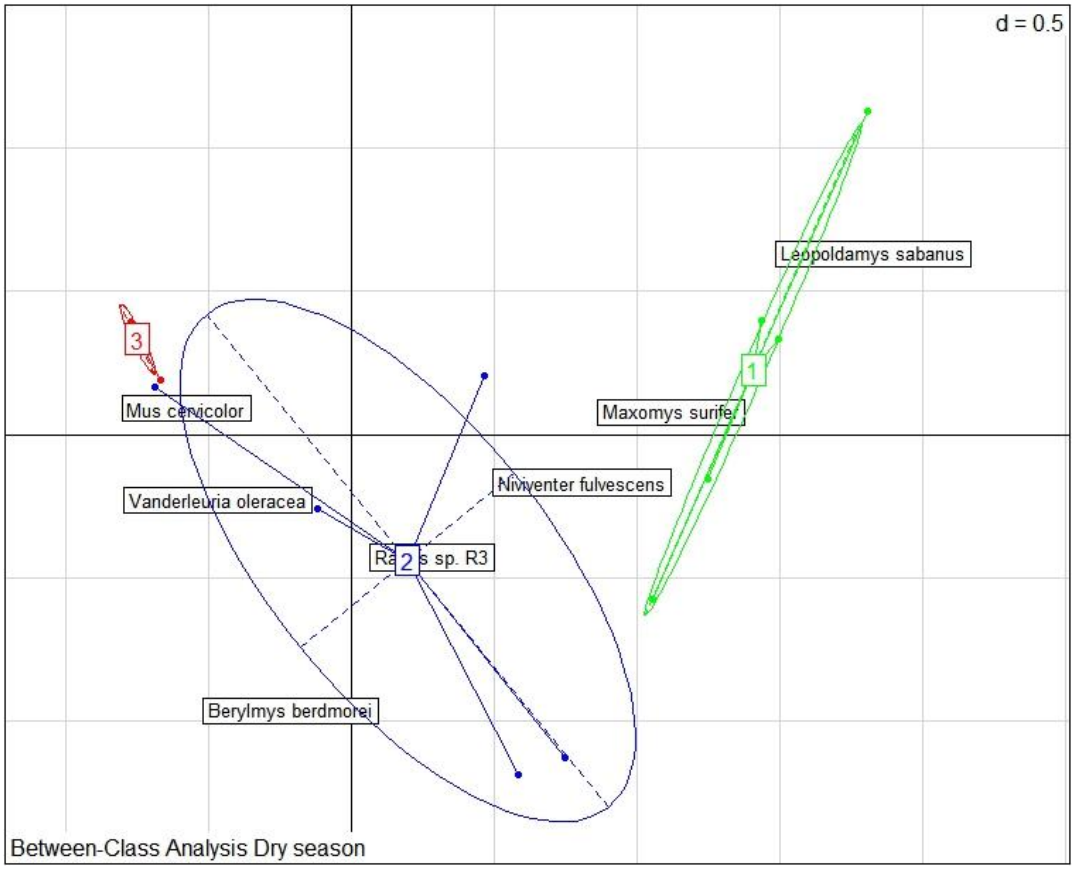

FIGURE 15 *Between-class correspondence analysis of species abundance among zones during the dry season The dots represent the sites, and colors the zones (Z1=intact forest green, Zone2=disturbed forest blue, Zone3=recently cleared field red). The sites are linked to the class-gravity, represented by the center of the ellipse.*

2.5. Zone preferences of species

Habitat specialization of rodent species was measured with the Shannon index, and represented in Figure 16, where species were regrouped by zones (based on the zone in which the highest number of captures were obtained for each species). A high Shannon index indicates a high habitat diversity of the species and thus a low habitat specialization (generalist species), whereas a low index indicates greater habitat specialization of the species (specialist species). Most species captured in intact forest and recently cleared fields, were specialist species, except for *Maxomys surifer* and *Mus cervicolor*, respectively, which were also present in high numbers in disturbed forest. All species found in disturbed forest were generalist species that is, able to adapt to multiple habitat types and were also found in intact forest and/or recently cleared fields.

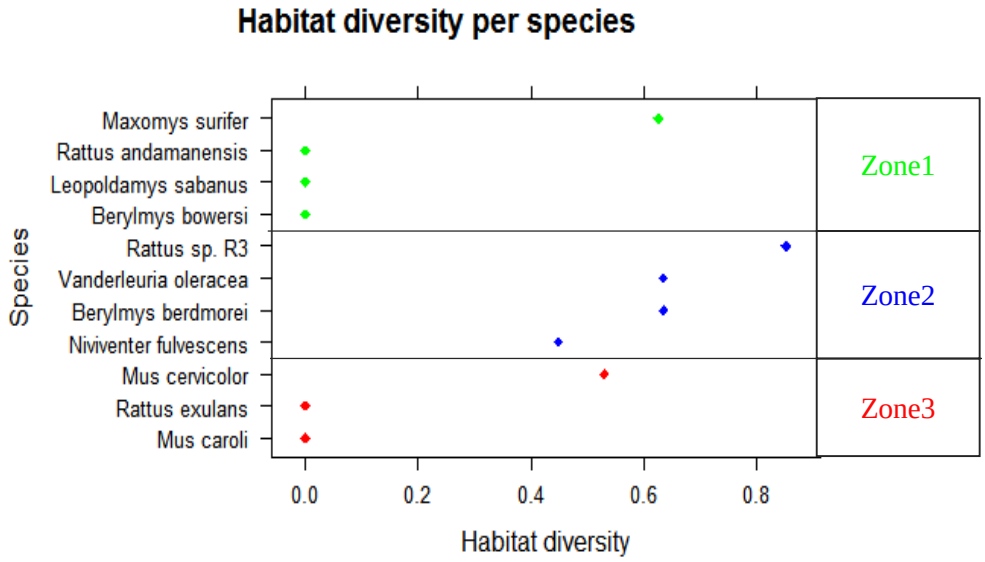

FIGURE 16 *Habitat diversity measured with the Shannon Index, per species*

Rodents are ranked according to their habitat diversity and grouped by zone in which the highest number of captures was obtained (rainy and dry seasons taken together).

(Zone1=intact forest green, Zone2=disturbed forest blue, Zone3=recently cleared field red)

For predominant species captured in at least two different zones (*M. surifer, R. sp R3* and *M. cervicolor)*, the effect of the season on the habitat (zone) was assessed using a negative binomial regression on the number of individuals from a given species, with season and zone as fixed effects and site as grouping variable. *Mus cervicolor* had a clear difference of distribution between disturbed forest and recently cleared fields depending on the season. There was a significantly higher abundance of *M. cervicolor* in recently cleared fields than in disturbed forest during the rainy season (coef. = 2.15 [1.15; 3.15] $P<0.001$), indicating a strong preference to the agricultural fields during the rainy season while there was no difference between the disturbed forest and recently cleared fields during the dry season.

Rattus sp.R3 was in significantly higher abundance in disturbed forest (Coef.=2.44 [0.92; 3.95], P=0.002) and in recently cleared fields (Coef.=1.7 [0.23; 3.27], P=0.024) compared to intact forest during the rainy season though there was no significant differences of abundance between zones during the dry season.

No zone or season effect was detected for *M. surifer.*

3. *Leptospira* infection

3.1. Pathogenic and Intermediate *Leptospira* PCR results

A total of 522 animals were tested and 63 were found positive with the *Leptospira* species real-time PCR, giving an overall prevalence of 12.1%. *Leptospira*-positive individuals were found in seven of eleven species and prevalence within species varied from 0 to 100%. A number of species (*Berylmys berdmorei, B. bowersi, Leopoldamys sabanus, Rattus andamanensis* and *Vanderleuria oleracea*) were rarely captured and the very low sample size did not allow estimating a reliable prevalence of *Leptospira* infection. Table 4 provides a summary of *Leptospira* prevalence (as determined by the *Leptospira* sp. real-time PCR assay) by rodent species, zones and season.

3.2. Pathogenic *Leptospira* PCR results

The pathogenic *Leptospira* real-time PCR protocol determined that 14 animals were positive for *Leptospira* spp. with pathogenic potential in humans (Table 3). Twelve of these strains were detected in the rainy season, all originating from *Mus cervicolor*, of which eleven were captured in recently cleared fields and one in disturbed forest.

In the dry season, two animals were positive for pathogenic *Leptospira*: one *Maxomys surifer* captured in disturbed forest and one *Berylmys bowersi* from intact forest. The sample size was too small to do further statistical analyses on the results from the specific-PCR.

TABLE 3 *Pathogenic Leptospira prevalence among rodent species and zones per season*

Species	Rainy season 2015			Rainy season Prevalence	Dry season 2016			Dry season Prevalence
	Intact forest	Disturbed forest	Recently cleared fields		Intact	Disturbed forest	Recently cleared fields	
Berylmys bowersi	-	-	-	-	50% (1/2)	-	-	**50% (1/2)**
Maxomys surifer	0/34	0/15	-	**(0/49)**	0/17	11,1% (1/9)	-	**3,8% (1/26)**
Mus cervicolor	-	2,6% (1/39)	4,8% (11/228)	**4,5% (12/267)**	-	0/36	0/33	**(0/69)**
Zone prevalence	0%(0/39)	1% (1/101)	4.2% (11/259)	**13.5%(12/399)**	3.8%(1/26)	1.6% (1/62)	0%(0/35)	**1.6% (2/123)**

TABLE 4 *Leptospira prevalence (from the Leptospira species essay) among rodent species and zones per season. (Zone1= Intact forest ; Zone2=Disturbed forest ; Zone3= Recently cleared fields) Numbers of positive samples over numbers of captured animal is (given in parentheses). The 95% confidence interval is given for the total prevalence per species in the rainy and the dry seasons. Species are ordered according to their total abundance.*

Species	Rainy Season 2015			Prevalence per species (Rainy)	Dry Season 2016			Prevalence per species (Dry)
	Zone 1	Zone 2	Zone 3		Zone 1	Zone 2	Zone 3	
Mus cervicolor	-	20.5% (8/39)	14.5% (33/228)	**15.4 ± 6% (41/267)**	-	(0/36)	3% (1/33)	**1.4 ± 5% (1/69)**
Rattus sp. R3	(0/3)	19% (8/42)	(0/21)	**12.1 ± 10% (8/66)**	20% (1/5)	23.1% (3/13)	(0/1)	**21.1 ± 21% (4/19)**
Maxomys surifer	2.9% (1/34)	6.7% (1/15)	-	**4.1 ± 10% (2/49)**	5.9% (1/17)	22.2% (2/9)	-	**11.5 ± 15% (3/26)**
Mus caroli	-	-	16.7% (1/6)	**16.7 ± 33% (1/6)**	-	-	-	**-**
Niviventer fulvescens	-	(0/4)	-	**0% (0/4)**	(0/1)	(0/1)	-	**0% (0/2)**
Rattus exulans	-	-	25% (1/4)	**25 ± 40% (1/4)**	-	-	-	**-**
Berylmys berdmorei	(0/1)	(0/1)	-	**0% (0/2)**	-	(0/1)	-	**0% (0/1)**
Vanderleuria oleracea	-	-	-	**-**	-	(0/2)	(0/1)	**0% (0/3)**
Berylmys bowersi	-	-	-	**-**	50% (1/2)	-	-	**50 ± 48% (1/2)**
Leopoldamys sabanus	-	-	-	**-**	(0/1)	-	-	**0% (0/1)**
Rattus andamanensis	100% (1/1)	-	-	**100% (1/1)**	-	-	-	**-**
Prevalence per zone	5.1% (2/39)	16.8% (17/101)	13.5% (35/259)	13.5% (54/399)	11.5% (3/26)	8.1% (5/62)	2.9% (1/35)	7.3% (9/123)

3.3. Sequencing results

A nested PCR was performed on all samples that were positive for pathogenic *Leptospira* to determine the bacterial species that were carried by the rodents. Of the fifteen samples that were positive for pathogenic *Leptospira*, an amplicon was successfully produced by the nested PCR for eight animals. These PCR products were then sequenced to determine the *Leptospira* species. Three different species of *Leptospira* spp. were identified (Table 5).

TABLE 5 *Pathogenic Leptospira species identified in the rodent species*

Rodent Species	**Season**	**Zone**	**Number of infected animals**	**Sample type**	***Leptospira* spp**
Berylmys bowersi	Dry	Intact forest	1	Urine	*Leptospira interrogans* serovar Icterohaemorrhagiae
Maxomys surifer	Dry	Disturbed forest	1	Urine	*Leptospira borgpetersenii*
				Kidney	*Leptospira weilii* serovar Topaz
Mus cervicolor	Rainy	Recently cleared fields	6	Urine	*Leptospira borgpetersenii*

L. borgpetersenii was identified from six mice. Two species were found in the same rodent, from two different samples (kidney and urine): *L. weilii* and *L. borgpetersenii* in a *Maxomys surifer* and finally *L. interrogans* was identified in *Berylmys bowersi*. Figure 17 represents the phylogenetic tree built using SeaView software with the BIONJ method. Reference *Leptospira* species sequences from GenBank were added to the alignment and phylogenetic tree.

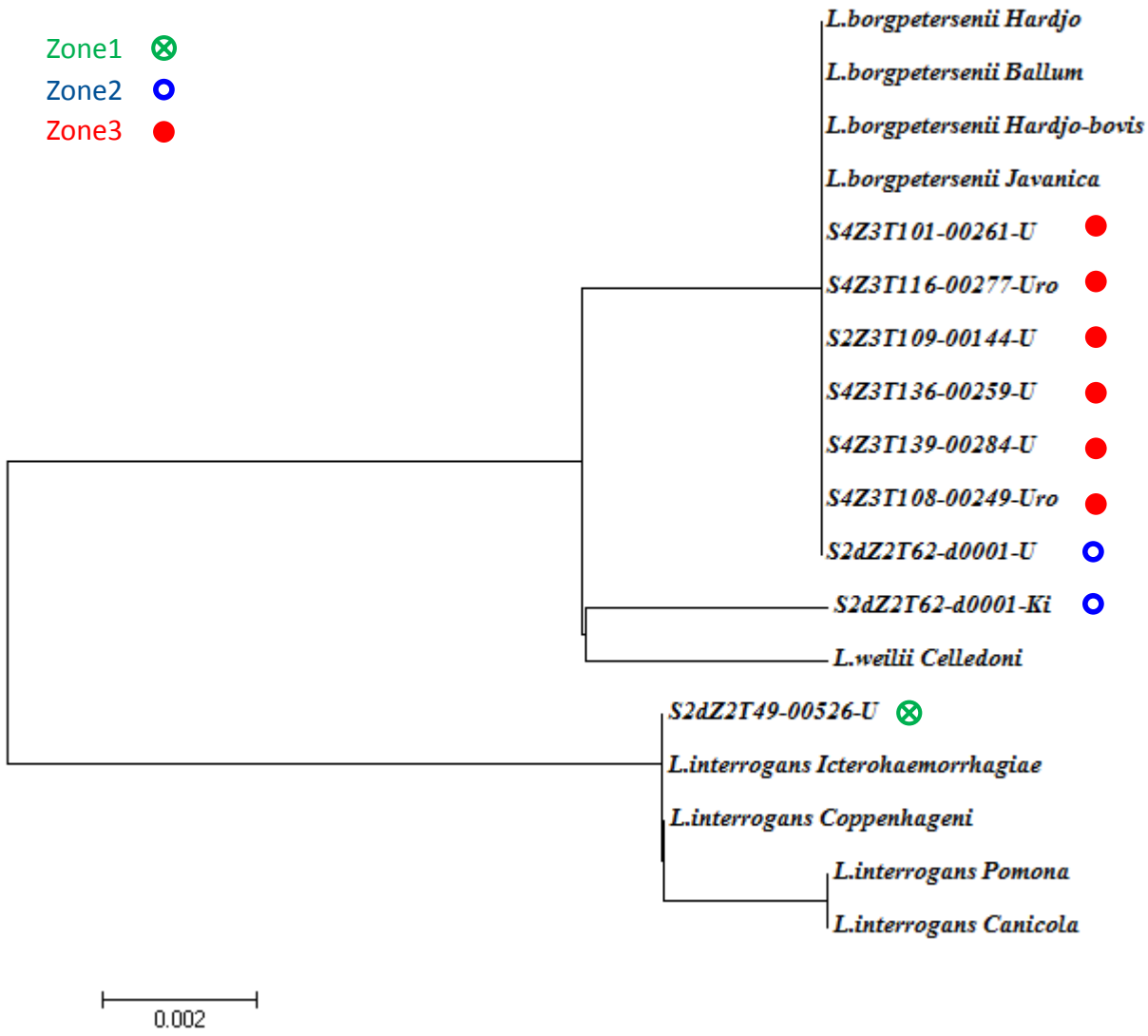

FIGURE 17 *Phylogenetic analysis for the rrs gene of Leptospira sp. isolated from rodents based on BIONJ method. Sequences from our samples are indicated with symbols per zone. (Zone1=Intact forest, green ; Zone2=Disturbed forest blue ; Zone3= Recently cleared forest Leptospira species from GenBank were added to the alignment for comparison.*

3.4. Statistical analysis on *Leptospira* infection

3.4.1. Univariate analysis

Univariate analysis of leptospiral infection revealed that season and sex significantly affected individual infection: males were significantly more likely to be infected than females (OR=2.27 [1.30-3.98], p=0.003) and rodents were more likely to be infected during the rainy season (OR=2.48 [1.17-5.24], p=0.013). However, there was no significant effect of rodent species or zones on the probability of infection. *Leptospira* prevalence was significantly different among sites (Chi2 P<0.0001). It is therefore important to conduct multivariate analysis with sites as a cluster effect, in order to take into account the matching of zones within sites in this study design.

The statistical association between the probability of infection and the season was not significant when stratified by site. Indeed, *Leptospira* prevalence in the dry and the wet seasons did not differ significantly within site except for one site which had zero infected rodents during the dry season.

3.4.2. Multivariate analysis

Species and zones were highly correlated and were therefore separately included in the models. The conditional logistic regression with clogit function (using STATA) with the explanatory variables: season and zone, or season and species as fixed effects, and site as a group factor gave no statistically significant relationships between the probability of infection and any of these variables (all P>0.10).

The probability of infection was significantly correlated to the sex, male were more likely to be infected than female (Adjusted OR=2.37 [1.30-4.31] P=0.005). The age (juvenile or adult) did not influence the probability of infection.

PART 6: DISCUSSION

PART 6: DISCUSSION

1. Rodent and deforestation

Tropical forests in Cambodia have been dramatically transformed in the past few years. The ecological and epidemiological consequences of this massive deforestation have not yet been assessed. Our data support the hypothesis that forest clearing and its conversion into agricultural fields engender manifest modification of rodent communities during these habitat changes.

The results of our study showed that deforestation clearly impacted the rodent species richness and diversity and greatly modified the overall rodent species composition. Species richness and diversity increased during the first steps of the deforestation process then decreased reaching a minimum in recently cleared fields. Species richness and diversity were greatest in disturbed forest, composed of species from both the forest and the agricultural fields. The disturbed forest rodent community appeared to be a mixing of species from the intact forest that persisted in the disturbed forest environment and of invading species from plantations. Such a mixing of species in the disturbed forest could favour spill over of pathogens between species, including *Leptospira*.

Bernard et al. (2009) observed a similar pattern with increased species richness and diversity of small mammal species in logged areas. Though they could not provide conclusive evidence as the study was conducted at a single local scale. Conversely, Wells et al (2007) observed contrary results with higher species richness and diversity in unlogged forest than logged forest in Borneo. In their study, this decline of diversity and richness was attributed to the reduction of rare species due to logging.

Our data identified a number of specialist species that were only found in the forest and seemed unable to adapt to the habitat disturbance caused by deforestation. However, one predominant forest species, *Maxomys surifer* persisted in disturbed forest. Conversely, some species were mostly found in agricultural land and could be considered synanthropic specialists. *Mus cervicolor* was found in the recently planted zone of all sites, regardless of the crop type (cassava, corn and rice field). *Mus cervicolor,* was also found in disturbed zones, possibly indicating an ability to invade disturbed forest early during the deforestation process. Another key species along the deforestation chronotone appeared to be *Rattus sp R3*, which was found in all three zones but with higher abundance in disturbed forest. Forest disturbance may provide an advantage to this species, allowing its population to grow.

Following early invasion of disturbed areas by synanthropic generalist species, crop plantation on cleared land resulted in the persistence of some generalist species (*Rattus tanezumi, Mus cervicolor*) and the replacement of others with synanthropic specialists, reaching high rodent densities.

Deforestation resulted in a significant increase of rodent densities during the process of land clearing. The highest rodent densities were detected in the recently planted fields. Though, their low richness and diversity indicated that the high density was due to a single species. There was a clear domination of the rodent community in recently cleared fields by *Mus cervicolor*.

Seasonality

Rodent densities in recently cleared fields appeared to be greatly influenced by the season, with high densities observed in the rainy season and a clear decrease during the dry season. In contrast, rodent diversity, richness and density in forest areas (intact and disturbed) did not vary greatly with the season, indicating some level of stability over the seasons. The change of total rodent density in agricultural zones was driven by *Mus cervicolor*, the most abundant species in this zone. In addition, there was a clear seasonal effect on *Mus cervicolor* zone preference. This species had a marked preference for agricultural fields during the rainy season but was found indifferently in the disturbed forest and the fields during the dry season. Additionally, field observations indicated that, even in agricultural zones, these mice were mostly captured in areas of denser vegetation in in the dry season. This suggests that the harsh conditions in the fields in the dry season (lower food and water resources) resulted in a retreat of this species into the nearby disturbed forest where food and cover may be more accessible. A study (Douangboupha et al., 2009) in Lao showed similar results but with migration of rodents from the fields to human settlements.

Rattus sp.R3 also displayed seasonal changes of distribution across zones, with higher abundance in agricultural zones in the rainy season (possibly driven by food abundance). This trend was also observed in previous studies in the region (CERoPath).

These seasonal movements among the deforestation stages and fluctuations in densities, influenced by agricultural activities, may have important consequences on the movement of *Leptospira* and other rodent-borne pathogens between zones, on their amplification, and on the exposure of humans to these pathogens. Our results allowed us to identify the species that could facilitate contact between forest and synantropic species. Thus *Maxomys surifer*, *Rattus sp.R3* and *Mus cervicolor* represented key species between the different stages of deforestation.

2. Leptospirosis

Surprisingly, we did not find significant variation of *Leptospira* prevalence between seasons. Previous studies have shown a seasonality of *Leptospira* in rodents in South East Asia with an increase of prevalence during the wet season (Cosson et al., 2014 ; Ivanova et al., 2012). Our data did not show such seasonality for *Leptospira* species prevalence. The low sample size limited our ability to assess this season effect on pathogenic *Leptospira* infection. A higher probability of infection was found during the wet season for the pathogenic and intermediate species (from the *Leptospira* sp. PCR). This difference of prevalence was however explained by only one site, which had no positive rodents during the dry season. The prevalence of the other four sites did not vary significantly between the two seasons.

Our analyses revealed that male rodents had a higher probability of being infected than females. This finding is consistent with previous studies (Cosson et al., 2014) . Possible explanations raised by these studies were that males are more susceptible to infection because of the cumulative effect of androgens reducing their immune competence and steroid hormones altering their behaviour (Klein, 2000). Males have a more aggressive behaviour and larger home-ranges, which promotes greater contact with other rodents, and may increase their risk of exposure.

Leptospira prevalence in rodents did not significantly vary across rodent species. Most rodent species were found to be infected with pathogenic or intermediate *Leptospira* species. Nevertheless, the disproportion of sampling size between species prevented us from highlighting any differences in prevalence between species. Human pathogenic species were found in three key rodent species: *Mus cervicolor*, *Berylmys bowersii* and *Maxomys surifer*. However, the low detection rate of pathogenic *Leptospira* strains meant that it was not possible to determine if these species were maintenance host and therefore if public health risks were associated with any individual rodent species. The presence of pathogenic *Leptospira* in species from intact and disturbed forest, though in low number, indicated that forestry activities could represent significant risk factor of human leptospirosis.

The PCR we used to genotype the pathogenic *Leptospira* species originated from a previously published method (Boonsilp et al., 2011). The alignment of the 481-bp *rrs* fragment of the eight samples showed the three different *Leptospira* species presented earlier but the differences seen at the nucleotide level were minimal. Blast analysis of these fragments split samples into three distinct *Leptospira* species. However, it is important to note that the sequenced fragment was

relatively short and somewhat conserved between species. Therefore, although it is a good indication of the presence of pathogenic *Leptospira*, in the future it may be more prudent to distinguish between *Leptospira* species by sequencing a larger fragment of the *rrs* gene or a more variable region of the genome.

Our findings are consistent with the circulation of zoonotic *Leptospira* species in rodent communities in SEA (Cosson et al., 2014). The three pathogenic *Leptospira* species we identified in rodents had previously been associated with human cases worldwide and two of them had been isolated in human in Cambodia: *L. interrogans* and *L. weilii* (Mueller et al., 2014). The use of serological classification of *Leptospira* in the different studies conducted in Cambodia limited our comparison of *Leptospira* species circulation in human and in rodents (Berlioz-Arthaud et al., 2010 ; Laras et al., 2002).

L. interrogans is mainly known to cause illness in humans and Icterrohaemorrhagiae serovar is often associated with generalized form of leptospirosis and with poorer prognosis (Segura, 2005). However, we could not identify with certainty to which serovar corresponded our sequence as there were minimal differences in the blasting result between serovars (similar alignment score, query cover percentage and identity percentage).

Though less common, *L. borgpetersenii* and *L. weilii* were also associated with human disease in the previous studies. Human cases of leptospirosis were diagnosed with *L. weilii* serovar Topaz in Australia and were often associated with cattle farming and banana plantations. Contamination of the environment by infected rodents or cows is thought to be the main route of transmission of *L. weilii* (Corney et al., 2008).

L. borgpetersenii is mainly a pathogen of cattle and can also causes infections in humans. In cattle, the infection poses significant problems from reproductive dysfunction to high mortality rates (Chideroli et al., 2016). This *Leptospira* species unusually requires strict host-to-host transmission (Bulach, 2006). Interestingly, *L. borgpetersenii* has the smallest genome among the pathogenic *Leptospira* species, and this genome reduction is believed to reduce the species capacity to survive in the environment. Therefore *L. borgpetersenii* is more likely to be transmitted by direct contact with contaminated body fluids (Xu et al., 2016).

Directly transmitted diseases can be modelled either with a density-dependant transmission, assuming that the rate of transmission increases with the host population density (Brearley et al., 2013 ; McCallum, Dobson, 2002) or with a frequency-dependant transmission, assuming that

transmission increases with the proportion of infected hosts within a population (Brearley et al., 2013 ; Begon et al., 2002). However, the separation between the two transmission models is not always straightforward and the transmission often follows intermediate patterns. Thus, we cannot ascertain which transmission model is followed by leptospirosis. Nevertheless, it is likely that higher density will increase contact and thus may increase opportunities for *L. borgpetersenii* transmission. Moreover, our data evidenced a higher prevalence in males which could be explained by their behaviour. A higher density is therefore likely to enhance the competitive behaviour of male rodents for territory and females, and consequently increases intra-specific contact and subsequent transmission of *L. borgpetersenii.*

Additionally, *L. borgpetersenii* was mainly found in *Mus cervicolor* whose density and distribution were greatly influenced by the season. This could result in seasonal fluctuations of *Leptospira* occurrence and seasonal increases of the risk of outbreak.

One *Maxomys surifer* from a disturbed forest zone during the dry season was also found to excrete *L. borgpetersenii*, the same *Leptospira* species that was found in mice during the rainy season. Because no *Maxomys surifer* from the intact forests were found to carry pathogenic *Leptospira,* it is possible that this species is not a usual host for *L. borgpetersenii*. Dispersion of *Leptospira*-carriers *Mus cervicolor* into disturbed forest zones could have resulted in an introduction of the bacteria into these areas and its transmission to other species such as *Maxomys surifer.* In Cosson et al study (2014), *L. borgpetersenii* was mainly found in *Mus* species (*Mus cervicolor*, *Mus caroli* and *Mus cookii*) and in *Rattus* species occurring in rice-fields or households (*Rattus losea*, *Rattus argentiventer*) and only in one *Maxomys surifer*.

However, we cannot conclude that this is the case as only one animal was found positive. Moreover, two different *Leptospira* species were identified in the same animal, one from a urine sample and the other from a renal sample. This could indicate a co-infection of the two *Leptospira* species. *L. weilii* that was found in the kidney but not in the urine, which could be explained by renal colonisation without excretion. *L. borgpetersenii* was extracted from the urine but not from the kidney as would be expected. It is also possible that the presence of multiple species is an artefact of the sequencing limitations described previously which would imply that only one *Leptospira* species was infecting this animal and better sequencing approaches are required to conclusively determine the infecting species.

Additionally, the presence of two species in the same area does not necessarily imply that there are contacts between them. Identifying intra- and inter-specific contact patterns in each zone would be essential to understand the transmission of *Leptospira* in these rodent communities. Nevertheless, the presence of *Leptospira* infected *M. cervicolor* in disturbed forest and recently converted agricultural land is a clear indication of an introduction of pathogenic *Leptospira* strains very early in the deforestation process. This highlighted that forestry activities could be potential risk factor of *Leptospira* transmission to human. Deforestation but also hunting and consumption of wild rodents could therefore increase the risk of leptospirosis.

3. Methodological considerations

3.1. Design

Previous studies found modification of rodent composition consecutive to forest fragmentation and deforestation (Wells et al., 2007 ; Charles, Ang, 2010 ; Umetsu, Pardini, 2007 ; Suzán, Armién, et al., 2008) with a similar pattern of reduction in specialist forest species and invasion of opportunist species. However, these studies compared logged versus unlogged forest and often compared sites that were geographically different. Morand et al (2015) stated that the observed rodent community composition changes were likely due to the geographical distribution of the species rather than habitat fragmentation, as it induced sampling bias. Matching three stages of the deforestation process within each site and allowed comparing the rodent species diversity and richness between the zones of the same site (and not between zones of different sites), providing more confidence that the observed differences are related to the deforestation process, and not site-to-site variation.

Landscape changes have temporal components as well as a spatial components and to better understand how they influence disease infection and prevalence, it is important to incorporate time in studies (Brearley et al., 2013). Longitudinal design are ideally suited to study temporal processes such as deforestation, although the required length of follow up often make this design impractical and too costly. In contrast, the fast rate of deforestation in Cambodia, and its unpredictable nature were major impediments in the planning and implementation of longitudinal studies. A chronosequence design was used as an alternative to longitudinal studies, substituting space for time. This method is often used in ecology to study vegetation or geological succession but has drawbacks related to the underlying assumptions (Johnson, Miyanishi, 2008). A critical assumption of chronosequence designs requires that each zone in the deforestation sequence only

differs by the stage (time) along the process and follow the same pattern. This assumption implies that abiotic and biotic conditions remained constant over the time span of the deforestation process and during our study and that all zones had the same pattern of change. In our study, the three zones (intact forest; disturbed forest; recently cleared forest) were matched in close proximity in the same geographical location and the recently cleared fields were always less than one-year old since last at the intact forest stage. The fast rate of deforestation and the simultaneous sampling of all zones of a site ensured limited changes of biotic and abiotic factors, other than those related to the deforestation process. This avoided regrowth and recovery of the original vegetation structure in the logged forest.

However, "intact forest" often had ongoing selective logging and it was difficult to find untouched forest areas. Thus, we were not able to cover the entire chronotone of deforestation, starting from the pristine forest, since these no longer exist in most regions of Cambodia.

3.2. PCR assay

In previous studies there have been a large number of genes targeted by PCR to detect *Leptospira* infection. The *rrs*, *gyrB* and *secY* genes are common for all *Leptospira* species including pathogenic and non-pathogenic species. The *ligA*, *ligB* and *LipL32* genes are restricted to pathogenic *Leptospira*. Therefore, PCRs targeting these genes detect pathogenic species only (Levett, 2001). This limited our ability to compare the *Leptospira* prevalence we found to other studies in SEA as the PCR methods often differed from one publication to another. Ivanova et al (2012) used the Mérien et al protocol (1992) that detected all *Leptospira* species including saprophytic species. Cosson et al (2014) performed a real-time PCR targeting the *LipL32* gene similarly to our specific-PCR. They found a prevalence of 4% in Mondulkiri province (out of 125 samples) and our overall *Leptospira* prevalence including both seasons was 3% (out of 522 samples).

Initially samples were screened using the real-time PCR method previously described (Thaipadunpanit et al., 2011 ; Slack et al., 2007 ; Smythe et al., 2002), targeting the *rrs*-gene. This allowed us to detect both pathogenic and intermediate pathogenic *Leptospira* species.

According to Thaipadunpanit et al. (2011) and Slack (2007) samples with a cycle threshold (CT) value below 40 should be considered positive. However, when we ran our analyses, we observed high levels of background for many samples with a CT between 35 and 40.We thus defined positive samples as having a CT below 35. Samples with a CT between 35 and 40 that displayed

a well-defined sigmoidal curve were considered potentially positive and will be retested. Comparing our results with studies that used the same PCR method was thus problematic as their cut off was possibly different.

The second real-time PCR from Stoddard et al. (2009) targeted the *LipL32* gene which is found only in pathogenic species. Three samples were positive with this pathogen specific PCR but were negative with the broad range *Leptospira* PCR. This is not entirely unexpected as the main advantage of a broad range PCR is to detect the largest panel of *Leptospira* species possible. The primers are designed to match a gene conserved in the widest range of species but if this sequence is not conserved in a species it will escape detection. Thus, this could explain why the three samples were identified using specific PCR methods but escaped the broad range detection. It is also possible that these PCR result were false positives as the fragments could not be amplified.

3.3. Barcoding

The individuals of *Rattus sp.R3* "species" identified by barcoding, were capture in all three zones during both the rainy and dry seasons. However, the taxonomic status of these rodents is unclear and has not yet been explored. It seemed several species could be included in this clade. Moreover, the morphology of some of the captured rats was quite different but no clear species identification was possible, thus they were grouped with *Rattus sp.R3* for the analysis, but this group may actually be comprised of multiple *Rattus* species. In similar studies (Morand et al., 2015 ; CERoPath, [sans date]) individuals from this clade were regrouped with *Rattus tanezumi* species, genetically the closest.

PART 7: CONCLUSION

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Leptospirosis is major public health problem in Cambodia due to its endemic and yet neglected status. Socioeconomic conditions, host density, climatic and environmental conditions and occupational habits of humans are determinants of the incidence and prevalence of the disease in humans. There is a need of transdisciplinary approaches to understand the complex role of these different determinants in leading to human infections. These approaches will need to include an epidemiological approach of *Leptospirosis* to identify the risk factors for exposure. There is a need to include ecological components as well: the *Leptospira* ecology to describe its survival in the environment, the host ecology to assess the transmission modes and describe the relationship between rodent population parameters and infection rates, and finally the ecosystem ecology to understand how landscape alteration affects all of the aspects above and its impact on human leptospirosis risk.

The current study, provided important information about the ecology, epidemiology and transmission of *Leptospira* in rodents in Cambodia. These findings may have important public health consequences, particularly in rural areas where the burden of leptospirosis is believed to be highest. Studies in these sites to evaluate the exposure of humans and domestic animals to leptospires are ongoing. These studies will investigate the seroprevalence of *Leptospira* in people engaged in land-clearing and planting to determine factors associated with a higher risk of *Leptospira* exposure. Water was collected from streams or puddles along random transects between traps of all three zones. Analysis of the water samples will provide information on the presence or absence of leptospires in the environment of each zone and on the *Leptospira* spp. strains if present, and their importance in Public Health.

Further work is needed in the public health sector to improve the knowledge of clinicians about the symptoms of leptospirosis and greater capacity for the laboratory detection of *Leptospira* in patients is urgently needed in Cambodia and the region.

ECOLE NATIONALE VETERINAIRE TOULOUSE

UNIVERSITÉ TOULOUSE III PAUL SABATIER

AGREMENT SCIENTIFIQUE

En vue de l'obtention du permis d'imprimer de la thèse de doctorat vétérinaire

Je soussignée, Mathilde PAUL, Enseignant-chercheur, de l'Ecole Nationale Vétérinaire de Toulouse, directeur de thèse, certifie avoir examiné la thèse de **RAMASSAMY Jill-Léa** intitulée **« Impact of deforestation on rodent distribution and on the prevalence of leptospirosis in Cambodia»** et que cette dernière peut être imprimée en vue de sa soutenance.

Fait à Toulouse, le 13 janvier 2017
Docteur Mathilde PAUL
Maître de Conférences
de l'Ecole Nationale Vétérinaire de Toulouse

Vu :
La Directrice de l'Ecole Nationale Vétérinaire de Toulouse
Isabelle CHMITELIN

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n'a plus aucun stage, ni enseignement optionnel à valider.

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APPENDICES

APPENDIX 1: Serological classification of *Leptospira* spp

Table A : Serogroups and serovars of pathogenic leptospires (*L. interrogans* complex)
SOURCE : Levett (2011)

Serogroup	Serovar(s)
icterohaemorrhagiae,	Icterohaemorrhagiae, copenhageni, lai, zimbabwe
Hebdomadis	hebdomadis, jules, kremastos
Autumnalis	autumnalis, fortbragg, bim, weerasinghe
Pyrogenes	pyrogenes
Bataviae	bataviae
Grippotyphosa	grippotyphosa, canalzonae, ratnapura
Canicola	canicola
Australis	australis, bratislava, lora
Pomona	pomona
Javanica	javanica
Sejroe	sejroe, saxkoebing, hardjo
Panama	panama, mangus
Cynopteri	cynopteri
Djasiman	djasiman
Sarmin	sarmin
Mini	mini, georgia
Tarassovi	tarassovi
Ballum	ballum, aroborea
Celledoni	celledoni
Louisiana	louisiana, lanka
Ranarum	ranarum
Manhao	manhao
Shermani	shermani
Hurstbridge	hurstbridge

APPENDIX 2: Serological and genomic classification of *Leptospira* spp

Table 1 : Genomic anc serologic classification of *Leptopira* spp
SOURCE : Bharti (2003)

Table 1. Classification of *Leptospira* species

Species	Serovar	Reference strain	Serogroup
Pathogens			
L interrogans	*australis*	Ballico	Australis
	bratislava	Jez Bratislava	Australis
	bataviae	Van Tienen	Bataviae
	canicola	Hond Utrecht IV	Canicola
	hebdomadis	Hebdomadis	Hebdomadis
	icterohaemorrhagiae	RGA	Icterohaemorrhagiae
	copenhageni	M 20	Icterohaemorrhagiae
	lai	Lai	Icterohaemorrhagiae
	pomona	Pomona	Pomona
	pyrogenes	Salinem	Pyrogenes
	hardjo	Hardjoprajitno	Sejroe
L alexanderi	*manhao3*	L 60	Manhao
L fainei	*hurstbridge*	BUT 6	Hurstbridge
L inadai	*lyme*	10	Lyme
L kirschneri	*bim*	1051	Autumnalis
	cynopteri	3522 C	Cynopteri
	grippotyphosa	Moskva V	Grippotyphosa
	mozdok	5621	Pomona
	panama	CZ 214K	Panama
L meyeri	*semaranga*	Veldrat Semaranga 173	Semaranga
L borgpetersenii	*ballum*	Mus 127	Ballum
	castellonis	Castellon 3	Ballum
	javanica	Veldrat Bataviae 46	Javanica
	sejroe	M 84	Sejroe
	tarassovi	Perepilitsin	Tarassovi
L weilii	*celledoni*	Celledoni	Celledoni
L noguchii	*fortbragg*	Fort Bragg	Autumnalis
L santarosai	*brasiliensis*	An 776	Bataviae
	georgia	LT 117	Mini
Genomospecies 1	*pingchang*	80-412	Ranarum
Genomospecies 4	*hualin*	LT 11-33	Icterohaemorrhagiae
Genomospecies 5	*saopaulo*	Sao Paulo	Semaranga
Saprophytes			
Genomospecies 3	*holland*	Waz Holland (P438)	Holland
L biflexa	*patoc*	Patoc I	Semaranga
L wolbachii	*codice*	CDC	

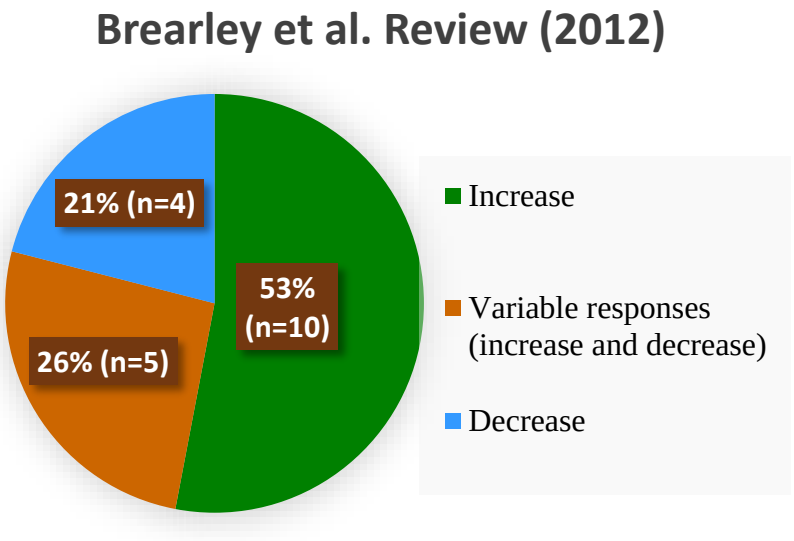
APPENDIX 3 : Host - serovars associations

Table : Typical reservoir hosts of common leptospiral serovars

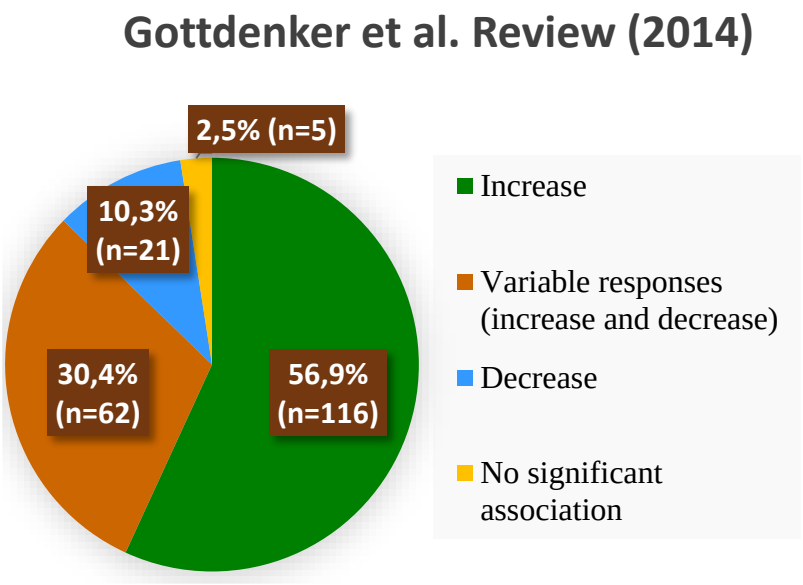
SOURCE : Bharti (2003)

Reservoir host	Serovar(s)
Pigs	*pomona, tarassovi*
Cattle	*hardjo, pomona*
Horses	*bratislava*
Dogs	*canicola*
Sheep	*hardjo*
Racoon	*grippotyphosa*
Rats	*icterohaemorrhagiae, copenhageni*
Mice	*ballum, arborea, bim*
Marsupials	*grippotyphosa*
Bats	*cynopteri, wolffi*

APPENDIX 4 : Summary of the trend of infectious disease response to land use changes from reviews



With n, the number of documented observational or experimental studies assessed in the review



APPENDIX 5 : Capture sites on the Cambodia Map

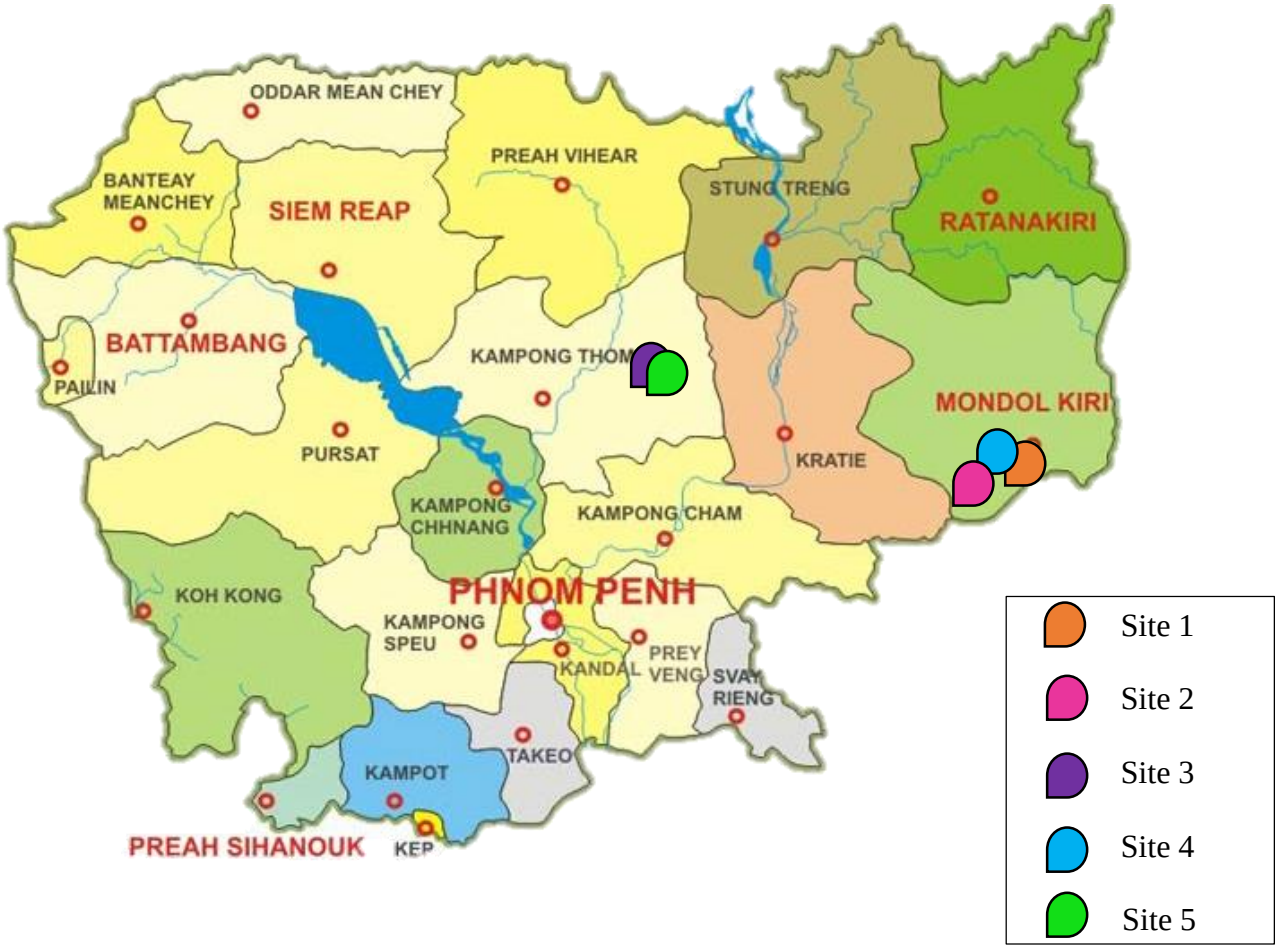
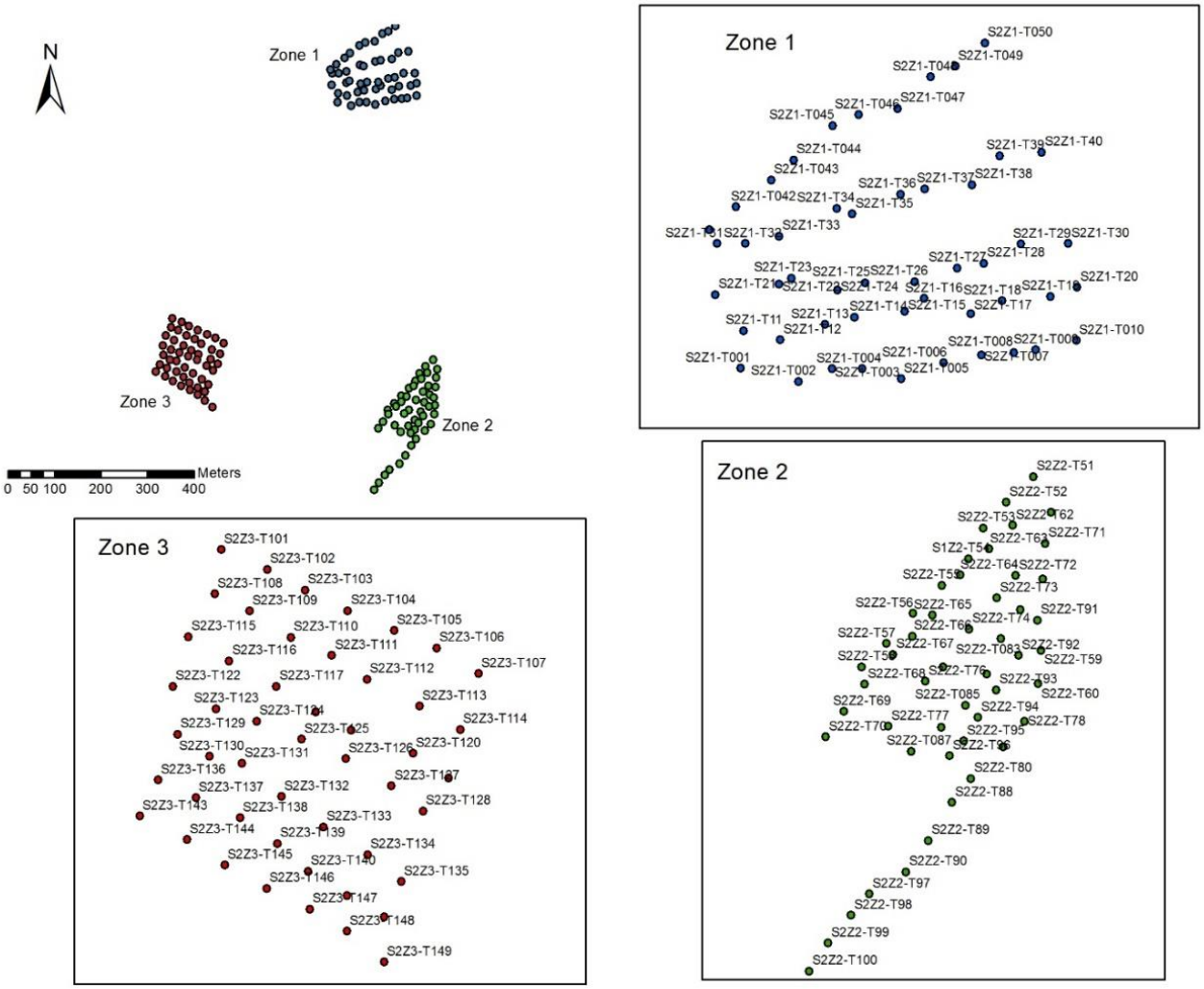


Figure 1: Cambodia Map with the five sites of capture

APPENDIX 6 : Gridline



Trapping gridline in Site 2 in Mondulkiri province. Each trap was located with a global positioning system (GPS) receive.

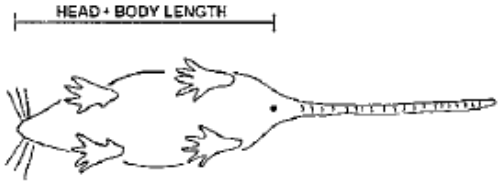
Picture 1:

Locally-made non-lethal Havahart trap.

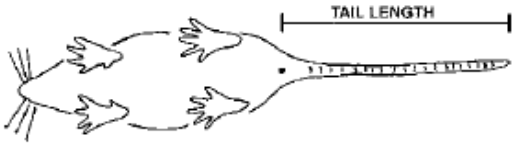
APPENDIX 7 : Animal identification

General			Male		Female		
Sex	Age	Species	Testicule score	Testicule length	Vagina	Teats score	Condition
☐ Male ☐ Female	☐ Adult ☐ Juvenile		☐ Partially Descended ☐ Non Descended ☐ Fully Descended		☐ Closed ☐ Open	☐ Indistinct ☐ Raised ☐ Lactating	

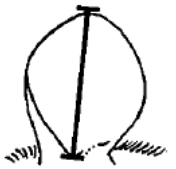
Measurements									
Left hind-foot length	Left ear length	Anal genital distance	Head + body length	Skull length	Tail length	Total (bag+ animal) weight	Bag weigth	Animal Weigth	Number injuries



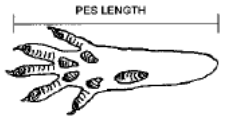
Measure the head+body length along the spine of the rodent from the tip of the nose to the middle of the anus.



Measure tail length from the middle of the anus to the tip of the tail.



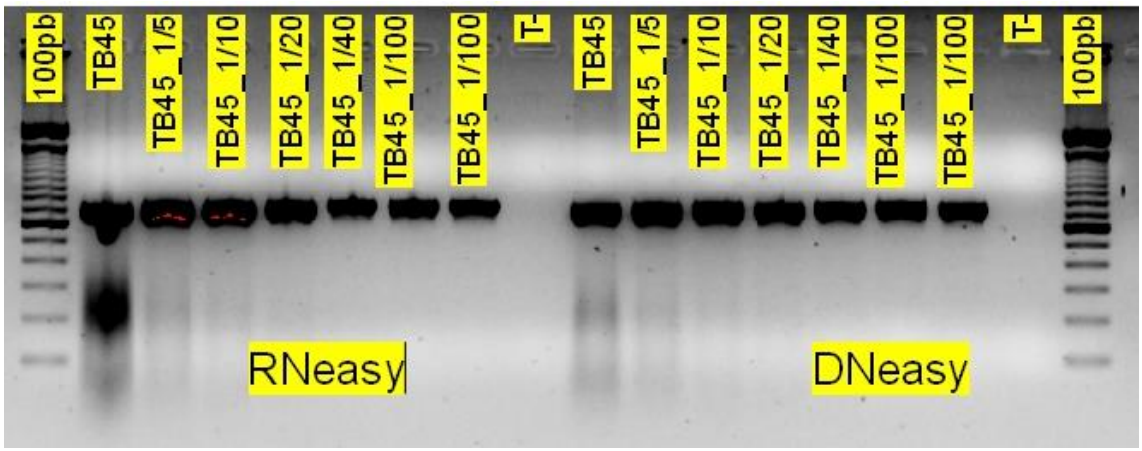
Measure the length of the ear from the bottom of the ear notch to the furthest point along the rim.



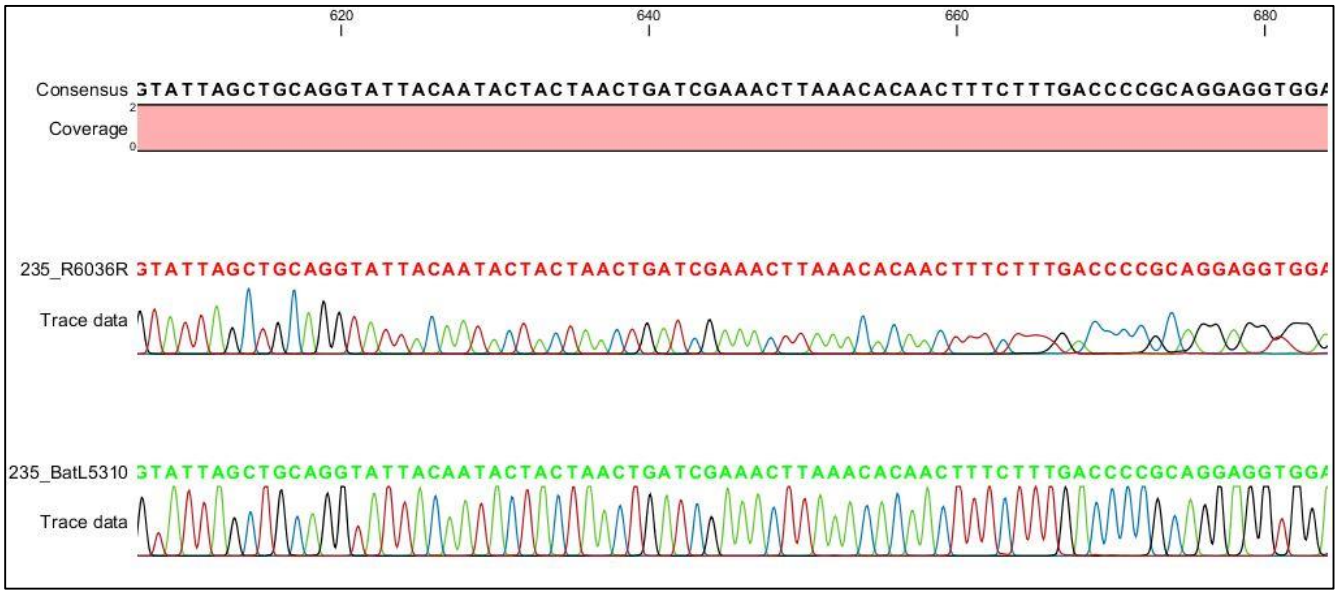
Measure the pes length from the base of the heel to the end of the toe pad on the longest toe (not including the claw).

General			Marking			
Site	Zone	Trap Number	Capture Class	Tag #	Fate	Animal ID
			☐ NC (new capture) ☐ RC (recapture)		☐ RT (Release with tag) ☐ D(Dead) ☐ E(escape without tag)	(Site-Zone-Trap-Tag# e.g. S1-Z3-T45-0005)

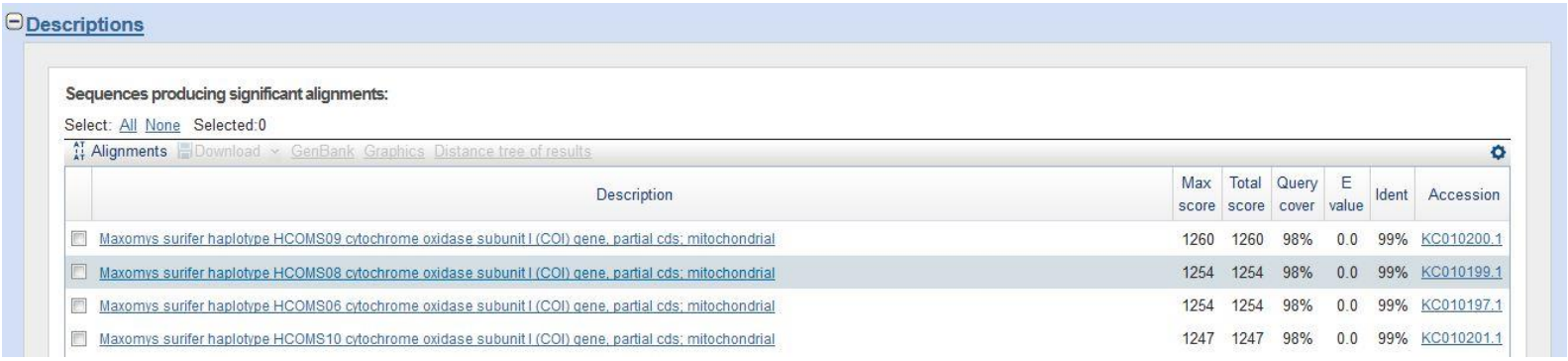
APPENDIX 8: Exemple of Barcoding protocol and result



Gel picture: detection of rodent DNA, diluted and extracted either with the RNeasy kit or DNeasy kit.



Sequence trimming, cleaning and analysis of a 700 bp fragment and alignment of the reverse and forward sequences on CLC Genomic.



Blasting results on NCBI

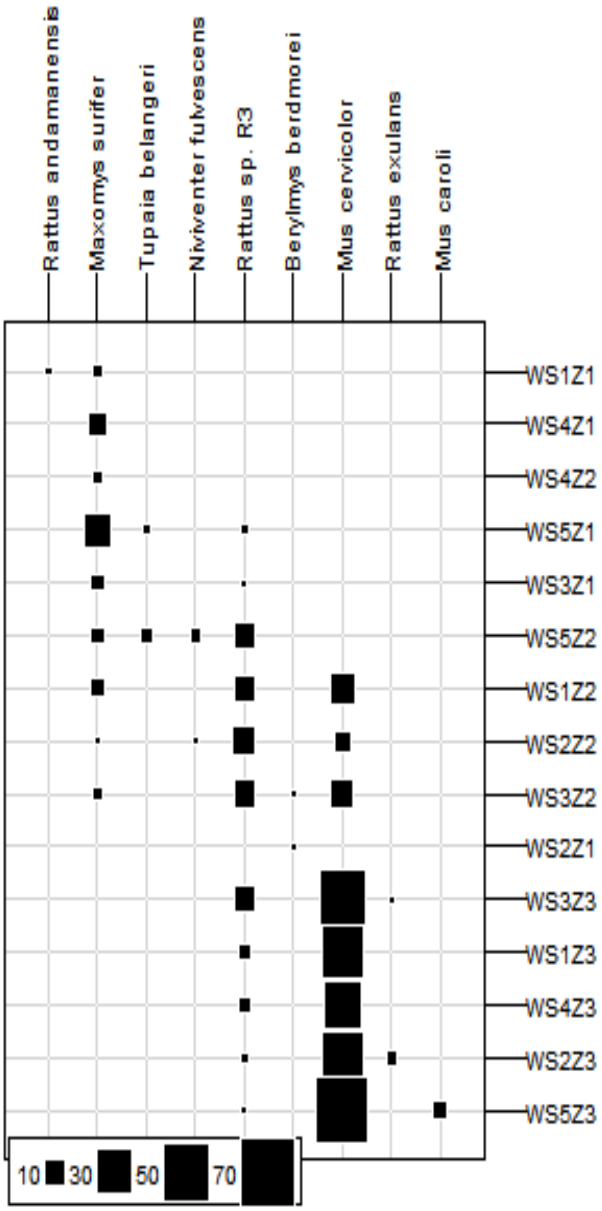
APPENDIX 9: Capture tables

Tables : Number of captured individuals per species per zone and per site for the Rainy and the dry season

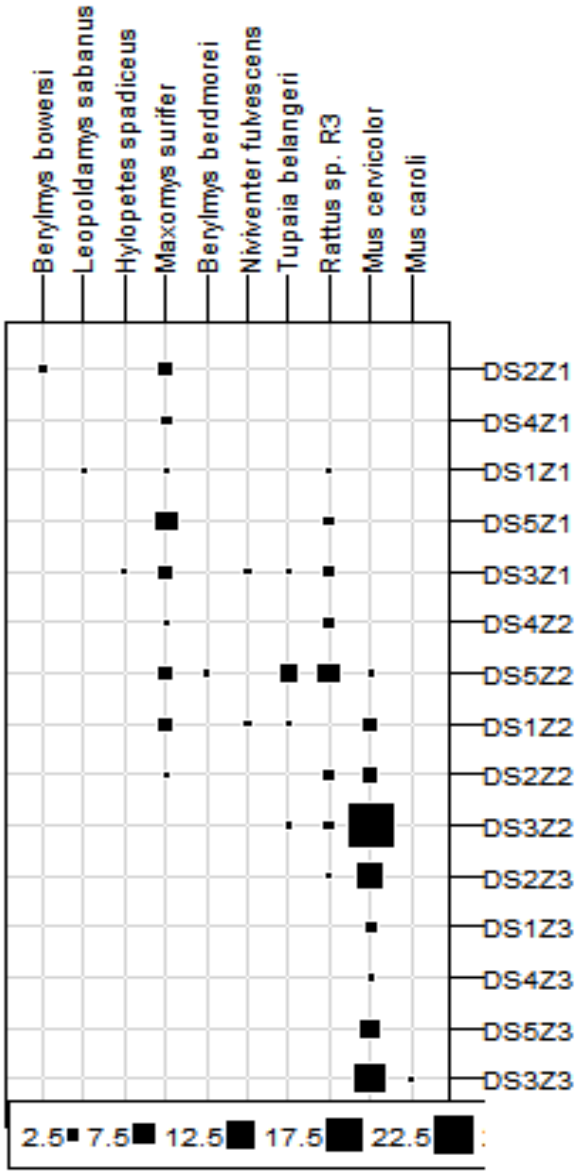
Species	Rainy Season 2015															
	Site 1			Site 2			Site 3			Site 4			Site 5			Total Rainy Season
	Z1	Z2	Z3	Z1	Z2	Z3	Z1	Z2	Z3	Z1	Z2	Z3	Z1	Z2	Z3	
Berylmys berdmorei	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	*2*
Maxomys surifer	3	5	0	0	1	0	4	2	0	9	3	0	18	4	0	*49*
Mus caroli	0	0	1	0	0	0	0	0	0	0	0	0	0	0	5	*6*
Mus cervicolor	0	20	40	0	6	39	0	13	49	0	0	35	0	0	65	*267*
Niviventer fulvescens	0	0	0	0	1	0	0	0	0	0	0	0	0	3	0	*4*
Rattus andamanensis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	*1*
Rattus exulans	0	0	0	0	0	3	0	0	1	0	0	0	0	0	0	*4*
Rattus sp. R3	0	10	4	0	12	2	1	11	11	0	0	3	2	9	1	*66*
Total	*4*	*35*	*45*	*1*	*20*	*44*	*5*	*27*	*61*	*9*	*3*	*38*	*20*	*16*	*71*	*399*
Tupaia belangeri	0	0	0	0	0	0	0	0	0	0	0	0	2	4	0	*6*

Species	Dry Season 2016															
	Site 1			Site 2			Site 3			Site 4			Site 5			Total Dry Season
	Z1	Z2	Z3	Z1	Z2	Z3	Z1	Z2	Z3	Z1	Z2	Z3	Z1	Z2	Z3	
Berylmys berdmorei	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	*1*
Berylmys bowersi	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	*2*
Leopoldamys sabanus	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	*1*
Maxomys surifer	1	3	0	4	1	0	3	0	0	2	1	0	7	4	0	*26*
Mus cervicolor	0	3	2	0	4	10	0	28	14	0	0	1	0	1	6	*69*
Niviventer fulvescens	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	*2*
Rattus sp. R3	1	0	0	0	2	1	2	2	0	0	2	0	2	7	0	*19*
Vanderleuria oleracea	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	*3*
Total	*3*	*7*	*2*	*6*	*7*	*11*	*6*	*32*	*15*	*2*	*3*	*1*	*9*	*13*	*6*	*123*
Tupaia belangeri	0	1	0	0	0	0	1	1	0	0	0	0	0	5	0	*8*

APPENDIX 10 : Relative abundance of each species per season



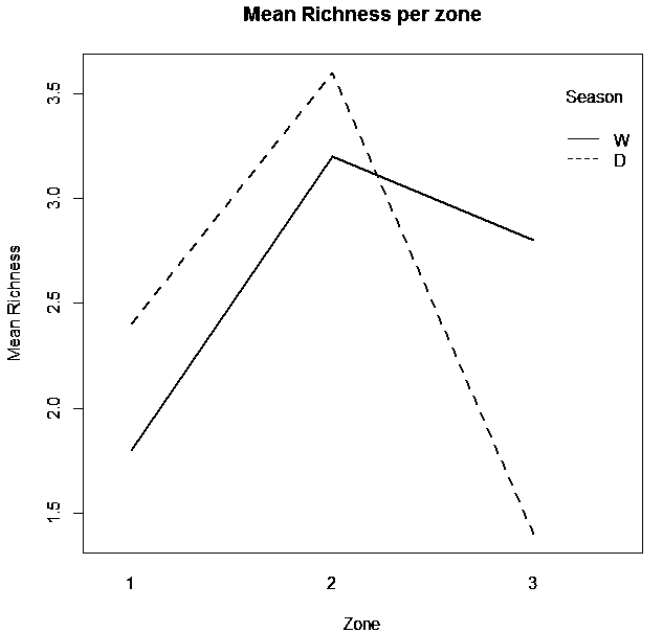
Wet Season



Dry Season

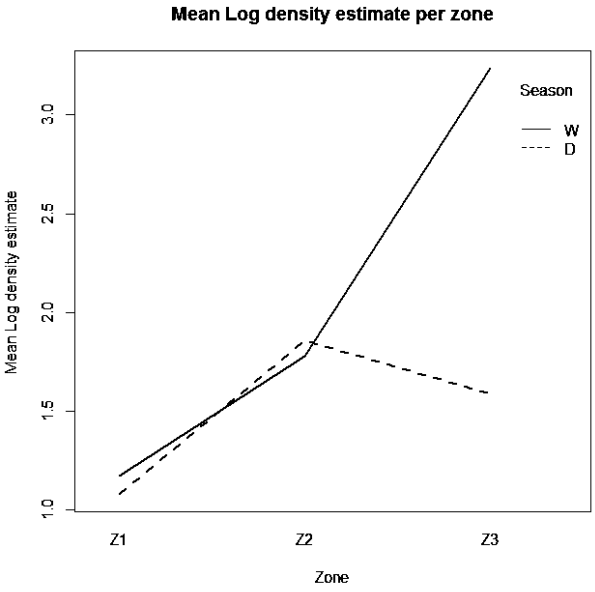
Relative abundance of each species in the different zones of capture per site and per season. The species are ordered (COA first axis).

APPENDIX 11 : Ecological indexes



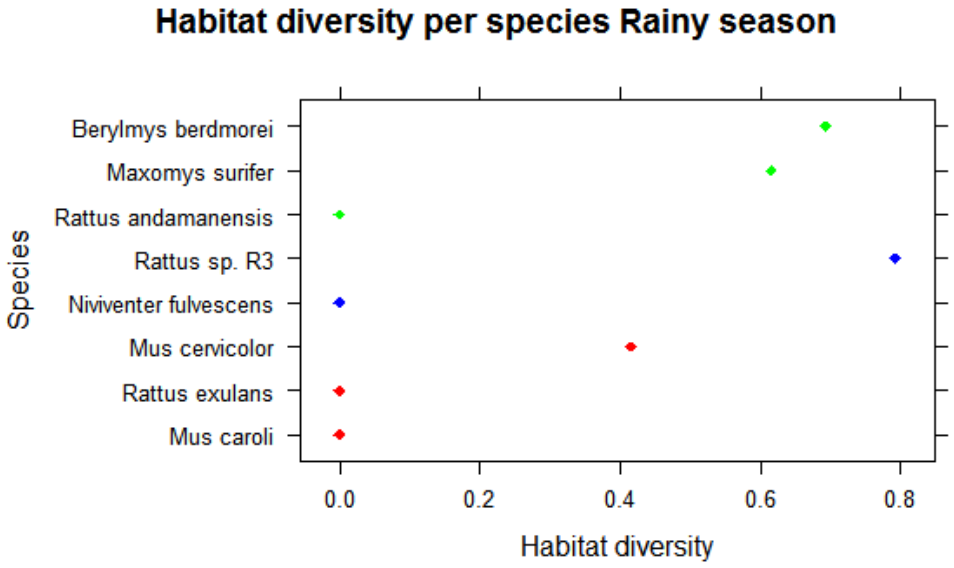
Graph 1 Mean species richness for each zone for the rainy season (full line) and the dry season (dotted line).
1: Intact forest
2: Disturbed forest
3: Recent fields

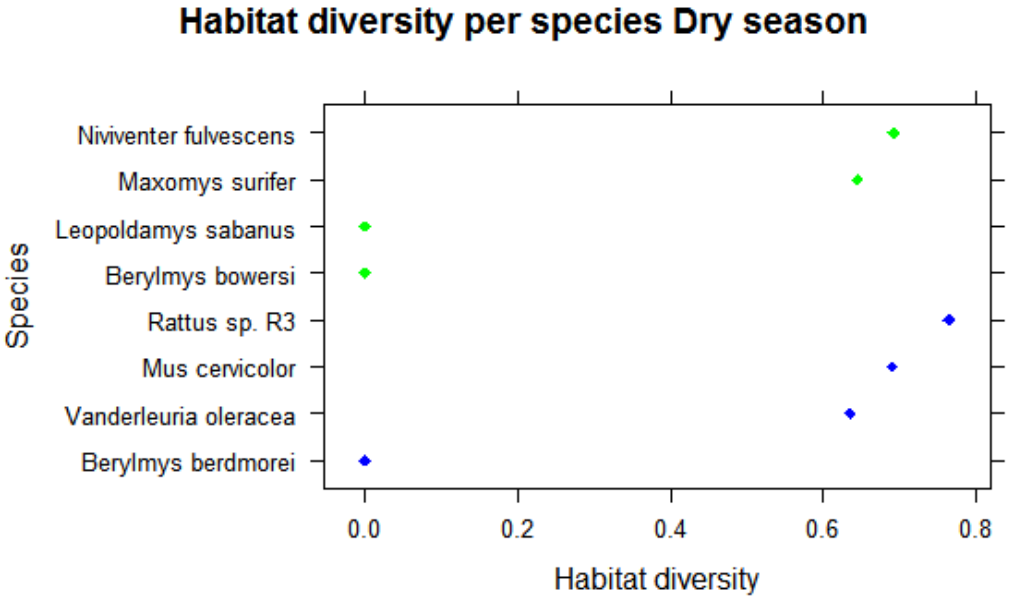
Graph 2 Mean species diversity (Shannon index) for each zone for the rainy (full line) and the dry seasons (doted line).
1: Intact forest
2: Disturbed forest
3: Recent fields



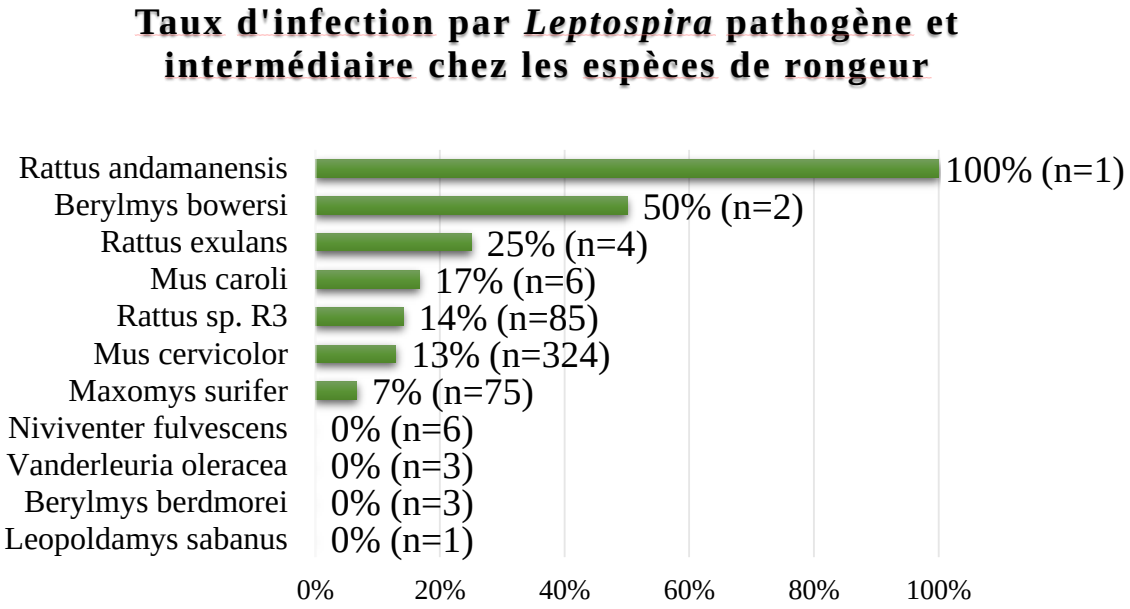
Graph 3 Mean of log transformed rodent densities for each zone for the rainy (full line) and the dry seasons (doted line).
1: Intact forest
2: Disturbed forest
3: Recent fields

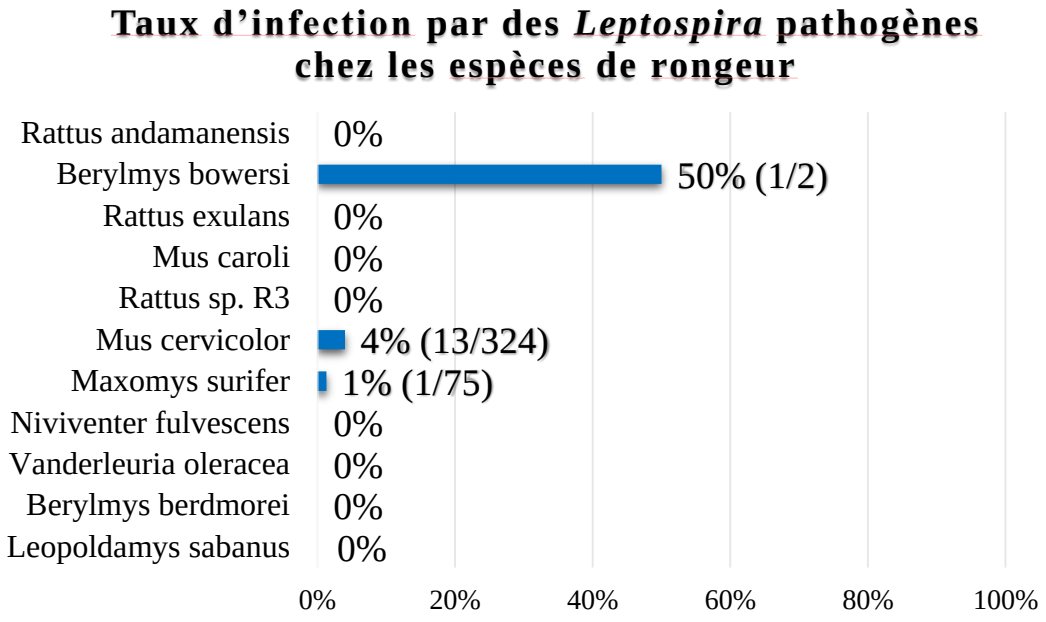
APPENDIX 12 : Habitat specialization per species per season





APPENDIX 13 : *Leptospira* prevalence





Funded by the European Commission under the INNOVATE programme

APPENDIX 14 : LACANET Project

LACANET project objectives

The LACANET One Health Surveillance and Laboratory Network project (also referred to as "LACANET") is an EU-funded project which brings together partners in the human health, wildlife health and animal health sectors to create capacity to survey, diagnose and understand the drivers of disease at human-animal-environmental interfaces.

The overall objective is to develop a bi-national Lao PDR-Cambodia One Health Surveillance and Laboratory Network that will enable both countries to:

1- Build capacity for surveillance and field investigation for zoonotic diseases:

For this to happen, we are training district, provincial and national wildlife and livestock health authorities in both Lao PDR and Cambodia to jointly conduct surveillance for zoonotic disease pathogens in vectors, wildlife and livestock populations using various sampling techniques.

We are also developing capacity to implement diagnostic testing for national priority diseases at the human-animal-environment interface between both human and veterinary diagnostic laboratories, using whenever possible similar techniques and standard operating procedures.

2- Improve laboratory capacity to detect zoonotic diseases

Laboratory experiments and analysis represent a significant part of the LACANET project, since we need to analyze all samples taken from the field. The Cambodian National Veterinary Research Institute (NaVRI) and the Lao PDR National Animal Health Laboratory (NAHL) regularly receive animal samples from suspected disease outbreaks from various Lao and Cambodian provinces for testing. Improving Lao and Cambodian laboratory capacity therefore appears as being critical. Therefore, the Institut Pasteur du Cambodge (IPC) and the Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (LOMWRU) are providing laboratory training to NaVRI and NAHL respectively from year 1 to year 4.

3- Improve national and regional cross-sectoral collaborations by establishing a One Health

Surveillance and laboratory network

Much of our efforts are designed to initiate lasting connections between One Health practitioners (field biologists and veterinarians, laboratory diagnosticians and medical microbiologists) within and between Lao PDR and Cambodia to promote knowledge transfer through exchanges, workshops and trainings, to encourage timely information sharing for effective and coordinated responses to zoonotic outbreaks. We are also hosting workshops on disease epidemiology and diagnostic techniques, across both animal and human sectors, and meetings to discuss One Health coordination as well as the economic and sociological aspects of these pathogens.

4- Conduct strategic research on two important drivers of disease emergence – Wildlife trade and land-use change:

We are investigating the role that land use change plays in disease dynamics by conducting surveillance for diseases with domestic and wild animal reservoirs, including Japanese encephalitis, leptospirosis and rickettsial diseases (as model disease systems) in vectors along a land use gradient, from pristine forest to industrial landscape.

We are also examining the role wildlife trade plays in disease emergence, including diseases such as rabies, anthrax, leptospirosis, typhus and trichinellosis, by conducting surveillance at high risk human-wildlife interfaces in wildlife market.

RAMASSAMY Jill-Léa

Titre : L'IMPACT DE LA DEFORESTATION SUR LA DISTRIBUTION DES RONGEURS ET LA PREVALENCE DE LA LEPTOSPIROSE AU CAMBODGE

Résumé :

La conversion de forêt en cultures agricoles entraîne des changements brusques et irréversibles des écosystèmes. De tels changements peuvent modifier la distribution des rongeurs et donc des pathogènes qu'ils hébergent. L'hypothèse que pose cette étude est que la déforestation modifie la population des rongeurs et favorise la transmission de leptospirose. L'étude se base sur un modèle de chronoséquence où trois zones à des temps différents de déforestation représentent trois étapes du processus de déforestation : forêt intacte ; forêt perturbée et champs récents. Les rongeurs sont capturés dans ces trois zones et testés par PCR en temps réel pour déterminer les individus excrétant des leptospires. Les leptospires pathogènes ont été séquencées et identifiées afin de déterminer le potentiel risque de leptospirose humaine au cours de la déforestation.

Mots-clés : Zoonose, Leptospirose, rongeurs, déforestation, écologie, épidémiologie

Title : IMPACT OF DEFORESTATION ON RODENT DISTRIBUTION AND ON THE PREVALENCE OF LEPTOSPIROSIS IN CAMBODIA

Summary :

Ongoing environmental changes, driven by deforestation, may affect the distribution of rodents and thus of the pathogens they carry. Our hypothesis is that the transition period from intact forest to cleared forest, represents an opportunity for an increase in the circulation of *Leptospira* spp. The main objective of our study was to assess the consequences of the changes occurring during the process of deforestation on leptospirosis. The design used for our study is a chronosequence sampling design. Rodents were captured in five localities in Cambodia with three zones characterizing different levels of forest degradation including: intact forests, ongoing clearing zones and completely cleared forests. The rodents were tested for leptospirosis by real-time PCR and *Leptospira* strains were identified in order to assess the potential risk for human health during deforestation.

Keywords : Zoonosis, leptospirosis, rodents, cambodia, land-use changes, deforestation, ecology, epidemiology